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CONNECTIVE TISSUE AND DISEASES OF CONNECTIVE TISSUE

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Conference Chairman and Consulting Editor

BERNARD M. WAGNER

CONTENTS

* This series of papers is the result of a conference on *Connective Tissue and Diseases of Connective Tissue* held by The New York Academy of Sciences on January 7 and 8, 1960.

INTRODUCTION

Bernard M. Wagner
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Following the elaboration of the concept "collagen diseases," an enormous investigative effort probing this idea has developed. Almost all branches of the biological sciences are currently involved in research related to collagen or connective tissue. From the physical chemist to the clinician, from the electron microscope to the whirlpool bath, the vast diversity of talents concerned with the common denominator, connective tissue, is apparent in the scientific literature. As the research expands and builds in complexity, it is vital that all lines of communication remain open and functional.

One of the purposes of this monograph is to bring together contributions from outstanding representatives in the field of connective tissue research. The contributors cover all phases, from a consideration of fiber formation and wound healing to reviewing immunological phenomena in connective tissue disease. This is an area under intensive investigation, and many aspects are in dispute. The use of new tools is briefly presented, and there is a review of host factors. This monograph contains stimulating material deliberately planned to evoke spirited discussion and to stimulate renewed effort in the field.

QUANTITATIVE STUDIES ON THE PRODUCTION OF ACID MUCOPOLYSACCHARIDES BY REPLICATE CELL CULTURES OF RAT FIBROBLASTS*

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Introduction

The acid mucopolysaccharide (AMPS) composition of the various connective tissues seems to be indicative of their specific differentiated states. Evidence that the distribution pattern of some seven or more different connective tissue AMPS (Meyer, 1957) is characteristic for each of many different connective tissues becomes increasingly impressive as work in this field continues (Meyer *et al.*, 1956; Hoffman *et al.*, 1957; Meyer *et al.*, 1958); in several tissues studied in detail this pattern changes materially with maturation or aging (Loewi, 1953; Loewi and Meyer, 1958; Hallén, 1958; Kaplan and Meyer, 1959). Furthermore, in connective tissue proliferation (Noble and Boucek, 1958; Slack, 1957; Jackson, 1957; Udupa *et al.*, 1956) and in many clinical and experimental states of hormonal and vitamin imbalance, connective tissue organization and AMPS content and composition frequently change together in a striking fashion (Dorfman and Mathews, 1956; Schiller and Dorfman, 1958; Asboe-Hansen, 1958, 1959).

The question of the sites and mode of formation of the intercellular substances of the connective tissue has been under discussion for the last century. Recent work with tissue culture systems has provided important new insights. Production of acid mucopolysaccharides by connective tissue cells *in vitro* was first demonstrated for cultures of synovial cells by Vaubel (1933), who sought to establish the source of synovial "mucin" (at that time the hyaluronic acid of synovial fluid had not been isolated or characterized; Meyer, 1957), and Vaubel's studies established the fact that the "synovioblasts" of the synovial membrane were responsible for its production. However, he reported that other types of connective tissue fibroblasts did not produce "mucinous" material. Further tissue culture studies, in particular those of Grossfeld *et al.*, 1955 and Grossfeld *et al.*, 1957 have offered strong evidence that tissue fibroblasts produce AMPS characteristic of the ground substance. The first of these studies showed that, in addition to cells from human synovium, fibroblasts of diverse species and from tissues of differing ages produced, *in vitro* in a biological medium, hyaluronidase-sensitive mucinous material over long periods of culture. The second study presented analytic data on the composition of some of these mucins, which proved to be predominantly hyaluronic acid together with some chondroitin sulfate. Other tissue culture studies

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confirming the production of AMPS by human synovial cells have been those of Kling *et al.* (1955), Castor (1957), Hedberg and Moritz (1958), and the tissue slice studies of Yielding *et al.* (1957). Production of AMPS by minced trypsinized tissues of chick embryo in a synthetic medium has also been reported (Berenson *et al.*, 1958).

A cell culture system, differing from conventional tissue culture in proceeding from a uniform cell suspension instead of from tissue fragments, would make possible investigations of quantitative cellular aspects of AMPS production unattainable with other methods. Cell suspensions of fibroblasts may be prepared by routine methods (Paul, 1959); with suitable modifications of technique, suspensions with enhanced cellular viability and other evidence of unimpaired function may be prepared (Harris, 1955*a*, *b*; 1959) and used to set up replicate cultures (Evans *et al.*, 1951; Paul, 1959), by the use of which growth and growth rates may be estimated from the desoxyribonucleic acid (DNA) content of representative cultures at intervals after setting them up (Healy *et al.*, 1954). In the present study, suspensions of osteogenic fibroblasts were prepared from cultures of newborn rat bone (calvaria) and from long-term cultures of similar material; replicate cultures were prepared from them and analyzed for growth as indicated and, in addition, for their total AMPS production and content in the cell-bound form.

Human synovial cells producing hyaluronic acid in culture continue to produce variable amounts of this substance when trypsinized and dispersed as subcultures (Castor, 1959). However, the capacity for AMPS production evidently may be decreased or lost during prolonged culture (Kling *et al.*, 1955; Castor, 1957, 1959; Hedberg and Moritz, 1958). Changes in the kinds and proportions of AMPS have been found where several different types are being produced (Grossfeld, *et al.*, 1957). Because of these findings, it seemed of interest to study the amounts, kinds, and proportions of AMPS produced by dissociated cells of a complex tissue such as bone, where all influence of their normal organized tissue milieu has been eliminated and they are permitted practically unlimited growth. Similar interest attaches to possible changes in the amounts and kinds of AMPS produced by cells as a result of prolonged *in vitro* growth; to this end, AMPS production by a three-year-old strain of fibroblasts was compared with that of similar cells recently isolated from the animal.

The idea that growth and differentiation are in some way reciprocal cellular functions often has been stated or implied (see Grobstein, 1959, pp. 484–486, for summary). To some degree, then, growth rate may reflect the state of differentiation of a cell; some overtly differentiated cells divide rarely or not at all. On the other hand, the rapid growth of cells in tissue culture apparently brings about a loss of, and reflects, differentiated properties, which may be prevented or reversed if growth is controlled (Willmer, 1958, p. 94 *et seq.*).

In so far as production and secretion of AMPS can be taken to represent a specialized function of a connective tissue cell, a system in which rate of AMPS production can be determined while growth rate is varied offers special advantages for study of this relation of growth to differentiated function. In addition to yielding quantitative information in an area of controversy, where

qualitative interpretations often have been advanced, such a system would permit detailed study of how the reproductive and synthetic activities of the connective tissue cell are related.

To evaluate aspects of fibroblast function as affected by changes in the cell culture environment, the following information was sought: (1) the amount and rate of AMPS production per cell under standard conditions, (2) the relationship between rate of AMPS production and rate of cell growth, (3) the kinds of AMPS produced by the cells under standard conditions, and (4) any differences in AMPS between cells recently isolated from rat tissues and cells of similar origin maintained in tissue culture for three years. Five variables were investigated for their effects upon growth and AMPS production:

(1) Diverse media promoting different rates of growth were compared for their effects upon AMPS production.

(2) "Crowded" cultures, where cell population approached a maximum and net growth ceased, were compared with uncrowded, rapidly growing cultures in amount of AMPS produced per cell.

(3) The *p*H of the medium was varied, since controlled increase in *p*H of the medium may inhibit multiplication of cultured cells for long periods while permitting a reduced rate of glucose utilization (Graff and McCarty, 1958).

(4) The effects of different concentrations of glucose and of glutamine were investigated because glucose and glutamine are reported to be specific intermediates for AMPS production in some systems, and it was desired to see whether production of AMPS per cell could be altered by varying their concentrations.

Resting streptococci utilize glucose and glutamine to produce hyaluronic acid (Lowther and Rogers, 1956), glucose is incorporated into AMPS hexosamine and glucuronic acid components without scission (Becker and Day, 1953; Roseman *et al.*, 1953, 1954; Schiller *et al.*, 1956; Roden, 1956*a*), and glutamine is reported to stimulate S^{35}-sulfate fixation in the AMPS of calf costal cartilage slices (Bostrom *et al.*, 1955; Roden, 1956*b*) and to stimulate labeling of AMPS with C^{14}-labeled glucose in calf cartilage and pig nucleus pulposus (Roden, 1956*a*, *c*).

The present investigation consists of 8 experiments reported in detail, together with additional or confirmatory data from a total of 58 experiments with groups of cultures prepared from rat osteogenic fibroblast suspensions. Additional information on the nature of the AMPS produced by the cells was obtained by preparing AMPS extracts from pools of used media and analyzing the material thus obtained by various chemical and enzymatic means.

Materials and Methods

Tissue Culture Techniques

Preparation of the Cell Suspensions

Fragments of periosteum-free calvaria of 1- to 3-day-old rats were explanted in thin plasma films in conventional Carrel flask cultures (Paul, 1959). For some experiments cultured tissue scraped from stock cultures (RB 1207/56), previously established from similar material, was subcultured in like manner.

After 10 to 14 days a suspension of the outgrowing cells was obtained from groups of Carrel flasks by a method of trypsinization modified from that of Harris (1955*a*). Cultures were washed and supplied fresh medium about 48 hours before the suspension was to be prepared. About 24 hours later the medium was again changed, and this 24-hour supernate (used medium) was saved for use in the final suspension; the fresh medium contained 20 μg./ml. of trypsin (Armour 2X-crystallized, salt-free); an equal amount of trypsin was added to the flasks a few hours before the flask contents were harvested.

To obtain the cells in suspension, flasks were rinsed with Earle's saline, and a warm solution of Armour's trypsin (75 μg./ml. in Earle's saline, *p*H 7.8) was added. About 10 to 12 min. of incubation at 37° C. sufficed to free explants and outgrowth from the flask floor. The suspension, broken up by rapid flushing in a pipette, was collected in sterile tubes kept in a jacket at 37° C. together with saline washes. Filled collecting tubes were left standing for 5 min. to settle, then all but the bottom 0.5 ml. of fluid was withdrawn and transferred to a small flask to which about 10 per cent of placental cord serum (PS) was added; the *p*H was brought to 7.1 to 7.3 by agitation of the flask contents in a stream of 7 per cent CO_2 in air. Settled-out residues were pooled, resuspended in fresh trypsin solution, and returned to the warm jacket for an additional 6 to 8 min. of digestion, and the resulting suspension was pooled with the first.

The final pool of suspended cells was subjected to repeated brief low-speed centrifugations (to a maximum of about 220 g), until examination of the supernate showed no appreciable number of intact cells. The thrown-down cells were then scraped from the sides of the centrifuge tubes with a sterile rubber policeman and resuspended in a medium consisting of "24-hour conditioned medium" (taken from the flasks and saved when the medium containing pretreatment trypsin was added) and Eagle's HeLa medium (Paul, 1959) in the proportions 1:2; *p*H was adjusted in a stream of CO_2 in air as required, and clumps of cells were strained out by passing the suspension through a stack of stainless steel gauze circles of increasing fineness up to 165 mesh. The total number of cells in the strained fluid was determined by counting a few drops in a hemocytometer and measuring the volume of fluid.

A final nutrient medium was prepared by adding fresh feeding medium and other components, as required, to the cell suspension; for most studies, the approximate final composition was: 5 per cent PS, 8 per cent beef amniotic fluid (BAF), 61 per cent Eagle's medium, 6 per cent Earle's saline, 20 per cent whole egg ultrafiltrate (WEUF) (Evans *et al.*, 1957), and glutamine at concentrations of from 0 to 225 μg./ml. In some studies a medium of the approximate composition of feeding medium (FM) No. 5 (TABLE 2) was used. Volume of the medium was adjusted to give from 30,000 to 150,000 cells per aliquot.

This suspension was then dispensed into previously gassed flasks (T-15 and, occasionally also, T-60 flasks) in measured aliquots; the suspension was constantly and gently stirred until dispensed to the flasks. Flasks were transferred to an incubator set at 37° C. and left undisturbed for at least 24 hours.

Most of the cells were well attached to the glass floors of the flasks after 11 to 12 hours; they flattened out quickly from the spherical form they had as-

sumed in suspension, and proceeded to elongate and to migrate; within 24 hours a number of cells were in mitosis. Originally the culture flasks were washed and refed within 24 hours; in more recent experiments flasks have been left for as long as 108 hours before feeding, during which time the original cell population increased at least fourfold.

Replication (uniformity of initial cell numbers among flasks of a set) improved during these studies as the techniques were developed and modified. In the last check on replication carried out, 5 aliquots of the suspension prepared for experiment 54 showed a coefficient of variation in their DNA content of 5.3 per cent.

Feeding and Harvesting Flasks Prepared from Cell Suspensions

Routine feeding. After withdrawal of used medium the flasks were washed once or twice with warm Earle's saline containing (usually) 2 to 5 per cent of placental serum; warm, freshly prepared medium (usually 1.50 ml.) was added; each flask was gassed with a warm CO_2-air mixture containing 5 per cent CO_2, and was returned immediately to a feeding table incubator at 37° C. The supernates (used medium) taken from 2 or more flasks were pooled and stored in the deep-freeze for later analysis, together with aliquots of the feeding medium.

Harvesting. When the cells and supernates were to be taken together for analysis, the pooled supernates and 2 saline washes for each group of flasks were centrifuged to sediment any residue of loosened cells or cell debris; the residue was washed with saline and saved, to be pooled with the harvested cell contents of that group of flasks. In some instances, as in the DD method (see under CHEMICAL TECHNIQUES) of AMPS extraction, the entire flask contents, supernates plus cells, were scraped and washed from the flasks and analyzed together.

The washed cell sheets remaining in the flasks were harvested in most cases by use of a buffered trypsin solution (usually 0.1 to 0.25 per cent of Difco 1/250 trypsin in Tris buffer, 1 ml./flask) added to each washed flask; after a few minutes at room temperature, when the cells could be dislodged from the flask floor by a gentle shaking, the suspensions and 3 washes of cold saline were collected and pooled in an ice bath, and stored in the deep-freeze until analyzed.

To see to what extent the amount of AMPS remaining bound to the cells was dependent upon 2 different procedures used for pretreating and rinsing the cultures before cell harvest, the following experimental procedure was employed in several instances. Groups of flasks to be harvested were arbitrarily divided into 2 sets. After supernates were removed, flasks of 1 set were briefly washed with warm saline before the cells were harvested; flasks of the other set were chilled in the refrigerator for 12 to 24 hours before the supernates were removed, and then the flasks were extensively washed with cold saline before the cells were harvested. Upon analysis for AMPS in the 2 sets, there was no significant difference between them in amount of cell-bound AMPS. The second saline rinse contained no detectable AMPS; the first rinse invariably contained approximately 5 per cent of the amount of AMPS found in the corresponding supernate.

Sources and Preparation of Tissue Culture Medium Components

Earle's balanced salt solution (Paul, 1959) was prepared, using Pyrex glass-redistilled water, and was sterilized by filtration through a Selas No. 03 filter. Concentrated stock solutions of other components, that is, glutamine, cysteine, and trypsin, were prepared with Earle's saline and sterilized by filtration through either a Selas or a Millipore filter. Such solutions were diluted further with Earle's saline or feeding media, or both, to required concentrations for use. Components were stored in the deep-freeze wherever possible.

Eagle's medium components were obtained as sterile concentrates.* These were added separately, as required, to sterile Earle's medium when the medium was prepared for each feeding. Glutamine† (cfp) was prepared as a concentrated stock solution and stored frozen. Whole egg ultrafiltrate was used.*

Beef amniotic fluid was drawn sterile from fresh intact placentas collected from local slaughterhouses. It was chilled and allowed to settle, or was centrifuged, and then was stored frozen.

Placental serum was prepared from blood of fresh umbilical cords collected in the delivery rooms of Presbyterian Hospital, New York, N. Y.; sterilized by filtration through Selas Nos. 01 and 03 porosity filters successively; and stored frozen. For dialyzed serum, the serum was dialyzed twice against 10 vol. of Earle's saline each time for a total of 60 to 72 hours at 3 to 4° C., then refiltered and stored frozen.

Chicken plasma was prepared from the blood of healthy young roosters without the use of coagulants; the blood was obtained either by heart puncture or by carotid artery cannulation. It was stored in paraffin-coated tubes in the refrigerator or the freezer. Chick embryo extract was prepared by extracting a coarse sterile brei of 9-day-old chick embryos with an equal volume of sterile Earle's saline for 2 hours at 2 to 3° C. and centrifuging. The clear supernatant fluid was stored frozen.

Penicillin and streptomycin were used routinely at concentrations of approximately 250 to 300 U. and 75 to 100 μg./ml., respectively.

Chemical Techniques

Methods of Extracting Acid Mucopolysaccharides of Supernates and Cells

DD method: hyaluronidase digestion, collection, and concentration of dialysates. This method, suggested by Alfred Linker as a modification of the methods of Weissmann *et al.* (1954), was used to obtain the products of enzymatic digestion of purified AMPS. Samples of 2 to 7 ml. were placed in dialysis bags fitted at one end with short stoppered tubes, and were dialyzed exhaustively against isotonic NaCl and acetate buffer (*p*H 5, *N*/10) until the collected concentrated dialysate gave no color in the Dische carbazole test for uronic acid. Testicular hyaluronidase,‡ 250 to 300 TRU/ml., was added to the samples in the bags and digestion was carried out according to Weissmann *et al.*, except that each

* Obtained from Microbiological Associates, Inc., Bethesda, Md.
† Obtained from California Corporation for Biochemical Research, Los Angeles, Calif.
‡ Supplied by Wyeth Laboratories, Philadelphia, Pa.

sample was dialyzed separately and simultaneously against several changes of their buffer until no additional carbazole color appeared in the dialysate. Collected dialysates were pooled and concentrated and assayed for uronic acid and, occasionally, hexosamine content. Concentration was effected in a rotary vacuum evaporator, after passing the pooled fluid through a Dowex-50 (hydrogen form) column to convert the buffer salts to volatile acids. Recovery of 80 μg./ml. of a hyaluronic acid-chondroitin sulfate (1:1) mixture, added to FM No. 5, was better than 90 per cent complete by this method.

While this method gave good yields and recoveries in the range of approximately 15 to 50 μg. of AMPS-uronic acid (AMPS-UA), it was considered of too low a sensitivity because low values were erratic; this was evidently due to the occasional presence of undesirable impurities.

Table 1
Media Used

Designation	Components (%)	AMPS-UA content μg./ml.
"Growth" media		
No. 5 (Biological)	20 PS, 50 BAF, 30 EBSS (+cysteine)	2.43
A-3 (Stock)	15 PS, 30 Eagles,* 30 BAF, 25 EBSS	2.14 ± 0.31(14)
E-5 (Low serum)	5 PS, 75 Eagles,* 20 WEUF	0.50 ± 0.13(14)
Maintenance (slow growth) media		
E-2	2 PS, 98 Eagles*	0.14 ± 0.05 (26)
DS 2/-	2 Dialysed PS, O-98 Eagles*	
Set-up medium		
Approximately E-5, contains also A-3 medium "conditioned" by previous growth of cells		

* Percent of standard Eagle HeLa formulation.

Abbreviations used: PS, (human) placental serum; BAF, beef amniotic fluid; EBSS, Earle's balanced salt solution; WEUF, whole egg ultrafiltrate; AMPS-UA, acid mucopolysaccharide uronic acid (determined by method of Bollet, Seraydarian and Simpson, 1957, as modified in text).

In 7 preliminary experiments involving cultures on biological growth medium (No. 5), supernate analyses were carried out by this method, together with DNA determinations, a total of 18 times. Specific production (see under *Results*) was calculated by dividing the found AMPS-derived uronic acid by the found DNA; mean specific production for these 18 determinations was 2.75 ± 0.18 (S.M.), with a range of 1.6 to 4.0 (micrograms AMPS-UA per microgram DNA). This mean value agreed well with values found using the PPP method (see below), as in experiment 50.

PPP method: perchloric acid-protamine precipitation of AMPS. The method of Bollet *et al.* (1957) for isolation of AMPS from serum was modified for these studies to permit the estimation of very small amounts of AMPS in tissue culture media, supernates, and cells. Bollet has since published a method for extraction of AMPS from tissues (Bollet, 1958) that is very similar. The method used here differs from Bollet's, chiefly in the milder and longer condi-

tions of alkaline hydrolysis and in its application to smaller amounts of material. Samples either of medium or of harvested cells in suspension were made $N/2$ to NaOH and left at room temperature under nitrogen. After 16 to 24 hours, samples were neutralized and 60 to 70 per cent perchloric acid was added slowly and with constant stirring to a final concentration of 5 per cent. Subsequent procedures were similar to those of Bollet. As previously reported by Bollet, the protamine present did not interfere with uronic acid determinations. Recoveries by this method were 85 per cent for approximately 25 μg. of a hyaluronic acid-chondroitin sulfate mixture, added to 2.5 ml. of supernate taken from cultures producing AMPS. Good reproducibility of single values will be evident from the standard errors given, where 3 or more determinations were made for a single analytic sample or equivalent samples in the experiments. Values for AMPS-UA present in the various culture media (TABLE 1) and their standard errors were calculated from the sum of replicate analyses carried out on aliquots of media from each experiment; variations for summed data were invariably higher than repeated analyses of the same sample, reflecting differences in AMPS content of different batches of serum and amniotic fluid used (Morris, unpublished data) as well as other possible systematic errors. Except where otherwise noted, extraction of AMPS was carried out by this method in the experiments to be described.

In some instances the AMPS-protamine complex in buffer was treated with 1/20 vol. of a 10 per cent sodium tungstate solution. The resultant white flocculent precipitate was centrifuged down after samples were left at 4° C. for from 2 hours to overnight; the supernate from the centrifugation was dialyzed against running tap water and distilled water for 48 hours. Then it was either (1) evaporated onto stainless steel planchets for counting of radioactivity (in experiment 58); the material then was redissolved in distilled water and shaken briefly with a few grains of Dowex-50 (hydrogen form), and its uronic acid content was determined, or (2) made $N/2$ to acetic acid and 5 per cent to sodium acetate and decanted into 3 vol. of 95 per cent ethanol. The precipitate that formed upon standing in the cold was centrifuged down, rinsed with 95 per cent, and absolute ethanol, and ether, and dried in a vacuum desiccator over phosphorus pentoxide. This material was used for chemical studies.

Methods of Estimating Acid Mucopolysaccharides and Their Components

Estimation of hexosamine. The method of Boas (1953) was used for occasional determination.

Estimation of uronic acid. The carbazole method of Dische (1947), as carried out by Bollet *et al.* (1957), was used with a slight change in the procedure for measuring the optical densities: samples were read in a Coleman Junior spectrophotometer (530 mμ) within 20 sec. of mixing with carbazole reagent in colorimeter tubes, and read again after 2 to 2½ hours; glucuronolactone standards and blanks were similarly read. Uronic acid content of samples was proportional to the difference in the 2 readings of optical density; a further small correction was applied for changes in the optical density reading of the blanks in the same interval.

A slight correction to the cell-bound AMPS-UA values was made for an unknown chromophore present in the crude trypsin solutions used to strip the cells from the glass at harvesting. The chromophore was found both in Difco 1/250 and Nutritional Biochemicals 1/300 trypsins. Determinations of AMPS-UA content were made on aliquots of the trypsin solution actually used. The substance usually was present at a concentration of about 0.5 μg. of UA equivalents per milliliter of 0.25 per cent solution.

Estimation of DNA

The original diphenylamine method of Dische at first was used with the 2-wave-length reading procedure of Zamenhof (1957) and his microadaptation, employing microcuvettes.* Later, the reagent modifications of Burton (1956) were used; this resulted in at least a sevenfold increase in sensitivity and a greatly increased stability of the colored complex. Purified DNA obeyed Beer's law in the range of 1 to 10 μg. under these conditions, and as little as 0.2 μg. of DNA could be measured accurately. Extinction measurements on 2.15-μg. aliquots of the same standard solution of DNA (hydrolyzed in 5 per cent perchloric acid and stored in the refrigerator) were reproducible to within 6 per cent (coefficient of variation, 5.8 per cent) in 43 separate determinations over a 10-month period. In practice, residues, after extraction of cell-bound AMPS by the PPP method, were obtained by the Burton procedure using 5 per cent perchloric acid solutions; a final extraction volume of 0.2 ml. was obtained. Duplicate 0.1-ml. aliquots of this extract were used for the DNA determinations.

Cytological and Histochemical Techniques

In the course of several experiments small rectangular cover slips (10 by 22 mm., cut from No. 1 Gold Seal cover slips) were cleaned and sterilized and affixed to the floors of T-15 flasks with a very small drop of a clotting mixture (chicken plasma plus embryo extract). When aliquots of a cell suspension were introduced into flasks containing such cover slips, "samples" of the flask population were available in those cells that attached themselves to the cover slips; these cells differed in no discernible way from cells in other areas of the same flasks. The cover slips were removed during maintenance or at harvesting of the flasks; upon removal, they were rinsed in warm saline and fixed and stained in various ways, or they were placed in wax-rimmed mounts in suitable culture medium and observed immediately by phase contrast, in the living state.

Fixation was in absolute methanol; in alcohol-formalin-acetic 18:1:1 (plus calcium); in formaldehyde vapor followed by 10 per cent formalin in Earle's saline and 1 per cent osmium tetroxide; or by freeze-substitution, according to the methods of Deitch and Godman (1955).

Staining was carried out with the periodic acid–Schiff (paS) reagent (Pearse, 1953), toluidine blue for metachromasia (Kramer and Windrum, 1955), Hale's colloidal iron (Pearse, 1953), or azure B (Flax and Himes, 1952).

* Pyrocell Mfg. Co., New York, N. Y.

Staining with paS was controlled by various extraction techniques, including lipid extractions and digestion with testicular hyaluronidase (150 to 200 TRU/ml.), saliva, and 0.5 per cent alpha-amylase.

Procedural Methods for Specific Experiments

Refer to TABLE 1 for medium formulations. Suspension culture flasks were T-15 flasks unless otherwise specified.

Experiment 50. Primary cultures: bone fragments grown 12 days on FM No. 5. Cell suspension cultures: 27 flasks with approximately 100,000 cells in each were prepared. Subsequent handling was as indicated in FIGURE 1 and TABLE 2.

Experiment 55. Primary cultures: bone fragments grown 15 days on medium A-3. Cell suspension cultures: 30 flasks containing approximately 45,000 cells each (0.24 μg. of DNA) were prepared. Maintenance medium used in feeding flasks of group B contained 2 per cent of dialyzed placental serum and the following Eagle's medium concentrations: for period III, 2 per cent; for periods IV and V, 10 per cent. Cover slips in 2 flasks of each group of 6 flasks were removed, washed, and fixed when flasks of that group were harvested. See FIGURE 2 for additional details.

Experiment 54. Primary cultures: subcultured from stock for 10 days on medium A-3. Cell suspension cultures: 36 flasks containing approximately 67,000 cells each were prepared; in addition, 6 double aliquots of cell suspension were delivered to centrifuge tubes for DNA determinations. The first changes of medium were at 83 hours for group B flasks and at 119 hours for group A flasks. Maintenance medium used in flasks of group B contained (in addition to 2 per cent of dialyzed placental serum) 2 per cent of Eagle's medium in periods II and III, and 25 per cent of Eagle's medium in periods IV to VI. Cover slips were present and were harvested as for experiment 55. Additional details are indicated in FIGURE 3 and the text tables.

Experiment 51. Primary cultures: subcultured from stock on medium A-3 containing glutamine at approximately 90 μg./ml. Cell suspension cultures: 36 flasks containing approximately 150,000 cells each, plus 4 T-60 flasks containing approximately 600,000 cells each were prepared. The suspension medium contained glutamine at approximately 150 μg./ml. After 96 hours of incubation, flasks received the experimental maintenance media indicated in FIGURE 4 and TABLE 5; 4 flasks were harvested at this time to establish initial values.

Experimental 52. Primary cultures: bone fragments cultured 14 days on medium A-3 containing glutamine at 90 μg./ml. per ml. Cell suspension cultures: 26 flasks containing approximately 40,000 cells each were prepared. After approximately 108 hours of incubation, the flasks were rinsed and were supplied maintenance medium containing 0, 2, or 10 per cent of Eagle's medium components (without glutamine); half the flasks of each group received glutamine at 10 μg./ml., as in experiment 51. Results after 2 additional incubation periods of 47 and 79 hours on this medium are indicated below.

Experiment 44. Primary cultures: subcultured from stock for 11 days on

medium A-3 containing glutamine at 310 to 325 μg./ml. Cell suspension cultures: 27 flasks containing approximately 100,000 cells each were prepared; the setup medium contained glutamine at approximately 310 μg./ml. The flasks were rinsed and refed 48 hours after setting up; E-5 medium containing glutamine at 225 μg./ml. was supplied. The flasks were incubated for 96 hours; the experimental medium then was supplied as indicated in TABLE 6.

Experiment 47. Primary cultures: subcultured from stock for 12 days on A-3 medium without added glutamine. Cell suspension cultures: 25 flasks with about 95,000 cells each and 4 T-60 flasks with about 380,000 cells each were prepared. The setup medium contained no added glutamine. The flasks were rinsed and were supplied E-5 medium without glutamine 48 hours after setting up. After 96 hours of additional incubation, the flasks were handled as in experiment 44 and as indicated in TABLE 7.

Experiment 58. Primary cultures: subcultured from stock on A-3 medium containing glutamine at 90 μg./ml. Cell suspension cultures: flasks were prepared with about 110,000 cells each. Approximately 70 hours after setting up, the flasks were rinsed and supplied E-5 medium containing glutamine at 150 μg./ml. They were incubated an additional 96 hours; at 72 hours, supplemental glucose, glutamine, and Eagle's amino acids were added in concentrated form at almost original concentrations. At 96 hours, 29 of the flasks were placed in an ice bath, rinsed twice with ice-cold Earle's saline containing 5 per cent of dialyzed placental serum and approximately 27 μc./ml. of added sodium-S^{35}-sulfate. More of the same medium was supplied, and the flasks were left in the ice bath. After approximately 4 hours the flasks were rinsed twice with chilled unlabeled medium and supplied warm medium of similar composition containing radiosulfate and glutamine at concentrations of 0, 30, or 150 μg./ml. Flasks were returned to the incubator for either 7 or 50 hours, when they were harvested. AMPS was extracted by a modification of the PPP method described under **Materials and Methods.** The repurified extracts, after drying on tared stainless steel planchets, had negligible weight (less than 0.2 mg.). The dried samples were counted on a Baird Atomic Geiger-Muller counter with a Mylar window at assumed infinite thinness. Counts were recorded sufficient to reduce counting errors to less than 1 per cent; each sample was counted at least twice. Counting rates were corrected for background. The samples were then redissolved from the planchets and their uronic acid content was determined.

Specific activity of the radiosulfate medium was calculated from the activity of the medium (2.775×10^6 cpm/ml.) and the inorganic sulfate content contributed by Earle's saline (103.2 μg./ml.); a correction factor (1.1450) was applied for decay of activity between times of analysis of samples and those of medium. The specific activity of the sulfate was estimated to be 30,800 cpm/μg. of sulfate.

Assumed "chondroitin sulfate uronic acid" was calculated by multiplying the observed radioactivity of the samples (in counts per minute) by the factor 6.6×10^{-6}; this factor was obtained by dividing the gravimetric conversion factor 2.035 (M.W. uronic acid/M.W. sulfate) by the specific activity (see TABLE 8).

Results

Realtionship Between Growth and AMPS Production

Rates of Growth and of Production of AMPS During a Feeding Period

It was found in preliminary experiments that the rate of production of soluble AMPS was not constant in the period following a change of medium. After an initial rapid rate, AMPS production tended to level off after 24 to 36 hours of incubation. Flask DNA content increased most rapidly in the period from 24 to 48 hours; this was also the period when bursts of mitoses could be observed in the cultures. After 72 hours, production of both addi-

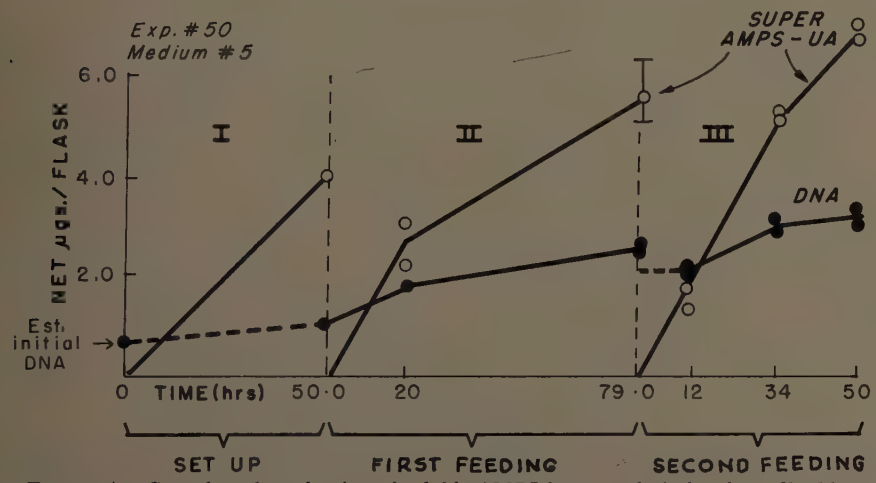

Figure 1. Growth and production of soluble AMPS by recently isolated rat fibroblasts in cell culture. See table 2 for details.

tional DNA and AMPS appeared to be slight but, if the period between feedings was extended an additional 24 hours to a total of 96 hours of incubation, a slight additional increment of AMPS usually appeared in the supernatant. In order to elaborate upon the preliminary findings, 4 experiments were carried out to investigate in detail the sequence of activities in the cultures following a change of medium (FM No. 5). Figure 1 and table 2 summarize the results of one of them (experiment 50). The cells were actively growing throughout this experiment. In the 50-hour setup period (period I), average flask DNA approximately doubled, increasing from an initial estimated 0.6 µg. to 1.13 µg.; during the subsequent 79-hour period (II), average DNA content more than doubled again; and in 50 hours of period III, there was a further DNA increase of approximately 32 per cent over the value at 12 hours. Visual observations of flask populations represented by high DNA values, such as the 3.00 µg. of period III, revealed that the cells were arranged almost entirely as a dense sheetlike monolayer; where the DNA content of the flasks was low, there was

a high incidence of free solitary cells and of cells with only part of their surfaces in contact with others in loose aggregates. Other experiments demonstrated that additional DNA sysnthesis after 72 hours was slight or absent when the cell population was moderately high. When the cell population was low, as when cultures were first set up, at least 2 cycles of DNA synthesis could occur in an extended interval between changes of medium.

At 20 hours after the first change of medium (period II), the cultures had produced about 47 per cent of the total AMPS they would produce in the entire 79-hour interval, while their average DNA content increased approximately

TABLE 2

RATES OF GROWTH AND OF AMPS PRODUCTION BY CULTURES OF FRESH RAT CELLS IN THE INTERVAL BETWEEN SUCCESSIVE CHANGES OF MEDIUM

(Experiment 50—Medium #5)

Feeding period	Hours from last feeding	Net supernate AMPS-uronic acid μg./flask	DNA μg./flask	Cell-bound AMPS-UA	Specific AMPS-UA production (μg./μg. mean DNA)	Rate constant spec. prod./hr.
I	50	3.98	1.13	—	—	
II	20	3.07	—	—		
		2.32	1.81	0.7	1.83	.092
	79	5.72 (±0.63)	2.62	0.4	3.10	.023
			2.50	0.55		
III	12	1.61	2.26	0.8	0.79	.066
		2.00	2.30	0.8		
	34	5.37	3.12	0.6	2.07	.062
		5.42	2.90	0.65		
	50	6.72	2.93	0.65	2.46	.029
		6.92	3.05	0.55		

Additional data for the experiment of FIGURE 1. Each value represents an analytical determination on the contents of 2 pooled T-15 culture flasks, except that the value with standard error is the mean of 6 such determinations.

60 per cent. A rate constant was calculated by dividing the increment of AMPS-UA produced in any interval by the mean DNA present in the interval and the interval length in hours. The initial rate of AMPS production in period II was 0.092 μg. AMPS-UA per mean microgram of DNA per hour. In the remaining 59 hours of period II, slightly more additional AMPS was produced than in the first 20 hours; DNA content increased an additional 41 per cent from the value at 20 hours. The rate constant for this interval was 0.023. In period III, 26 per cent of the AMPS-UA to be produced over a 50-hour interval had appeared after 12 hours, with a rate constant of 0.066. In the second interval of 22 hours, the rate constant of AMPS production did not change materially, while average DNA content of the flasks increased 32 per

cent;* a further 53 per cent of the total AMPS-UA was produced in this interval. The third (16-hour) interval was characterized by a decreased rate of AMPS production (rate constant, 0.029) and no further increase in DNA content of the flasks.

A summary of the growth and AMPS production of periods II and III: in the 79 hours of period II, DNA increased 126 per cent, AMPS production increased at least 1.45 times over its estimated value in period I, and 3.1 μg. of AMPS-UA was produced per microgram of mean DNA over the entire period; this ratio was termed the specific production per period. Specific production over the 50 hours of period III was 2.5 μg./μg. mean DNA, and DNA increase was 32 per cent. It is of interest that, in the 2 periods, initial rate of AMPS production was proportional to amount of growth occurring.

A small amount of "cell-bound" AMPS, which remained with the cells after washing, appeared to increase in the first intervals after each feeding, and to decrease slightly thereafter, as the concentration of AMPS in the supernate increased. The trend of the results of three other experiments analyzed by another DD method was similar.

Results of this and other time-study experiments indicated that soluble AMPS appeared in the medium in detectable amounts within 4 hours after a change in medium; that rate of AMPS production continued undiminished during intervals of DNA synthesis; and that production of AMPS fell off and synthesis of DNA stopped later in a feeding period. A change of medium appeared to trigger this cycle; the cycle was repeated after each additional change of medium, with some differences as the cells became crowded.

AMPS Production as Affected by Growth-Sustaining Properties of the Medium

Growth rate and AMPS production were compared in different media: after one or two periods of growth upon a standard medium, flasks of a series were divided into groups, some receiving other media. Subsequent changes in DNA content and AMPS production of flasks in the different groups were followed and compared. Results of an experiment with "fresh" rat cells are given in FIGURE 2 (experiment 55).

In little more than 7 days after 1 period each on setup medium and growth (E-5) medium, flask DNA content increased a *minimum* of 7.3 times. Production of AMPS in the supernate increased in this period from 1.50 μg. of AMPS-UA in period I to 2.4 μg. in period II. The flasks were divided into 2 groups and transferred to different media at the end of period II.

At the end of period III, 96 hours after random grouping, flasks that received growth medium (A-3) had further increased their DNA content 3.1 times, and their production of supernate AMPS had increased 2.9 times over values found for the previous 96-hour interval. Specific production of AMPS was 1.83 μg.

* The calculated increase of DNA in period III is based on the 12-hour value for DNA rather than on the value at the end of period II; the latter value is considered too high because of dead or dying detached cells containing traces of DNA, which were routinely recovered from supernates and washes by centrifugation and combined with the harvested cells for subsequent analysis (see **Materials and Methods**).

AMPS-UA per microgram DNA. In flasks supplied maintenance medium (DS2/2), period III saw no increase in flask DNA content and a sharp decrease in production of AMPS to 27 per cent of its value in period II; specific production of AMPS was 0.46 μg. per microgram DNA, one fourth of the specific production of the other group on growth medium. In flasks continued on a maintenance medium (with slightly higher concentrations of Eagle's medium) for 2 additional periods (IV and V), production of AMPS decreased further to 0.2; average DNA content increased slightly more than one third in the 6-day interval.

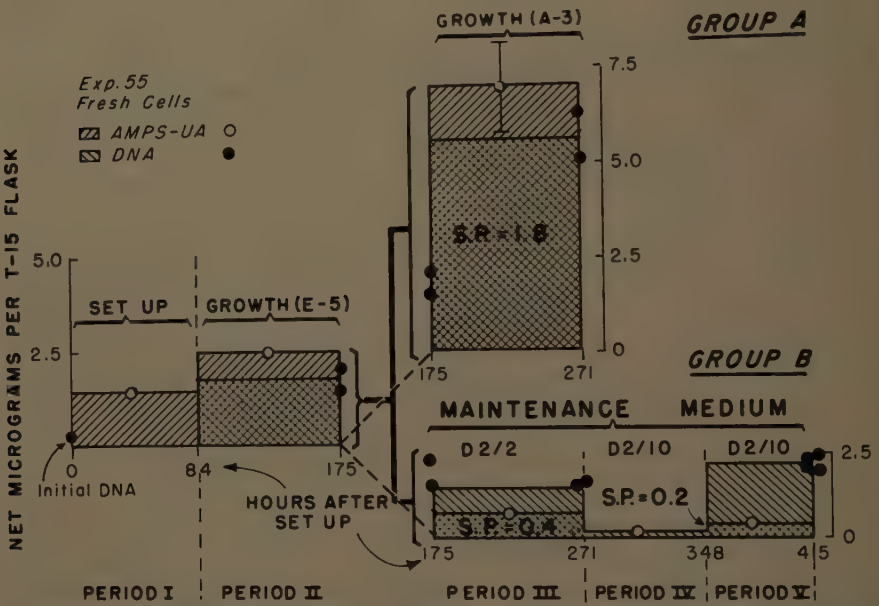

FIGURE 2. Growth and production of soluble AMPS over successive feeding periods by cell cultures of rat fibroblasts recently isolated. Cultures were grouped at random and transferred to either growth or maintenance medium at the end of period II. Differences in growth and total and specific production of AMPS (*see text*) 96 hours later are obvious. Slight growth and production of AMPS occurred on maintenance medium during two additional periods. Except where shown, standard errors for replicate AMPS determinations were small and were omitted for clarity.

Results of an experiment with stock rat cells, in which growth and maintenance media were compared, are summarized in FIGURE 3 (experiment 54). In flasks of group B, transferred to maintenance medium 83 hours after setting up and carried on maintenance medium for 5 successive periods, there was probably no growth from the end of the setup period (I) through period III, when the first flasks were analyzed. AMPS production per flask remained essentially constant over these first 3 periods. Flasks harvested at 29 hours of period III gave a value of 0.9 for the specific production and a value of 0.030 for the rate constant of AMPS production. There may have been a small amount of additional production during the remainder of period III, so that specific production at 74 hours (based on DNA values at 29 hours) was approximately 1.0.

During 3 subsequent incubation intervals on maintenance medium of somewhat increased Eagle's medium content, production of AMPS per flask in group B increased progressively and ultimately reached approximately twice the value for period III. Over this additional 9-day interval, average flask DNA content also increased to slightly more than twice its previous value; specific production in the final period (VI) therefore remained at approximately

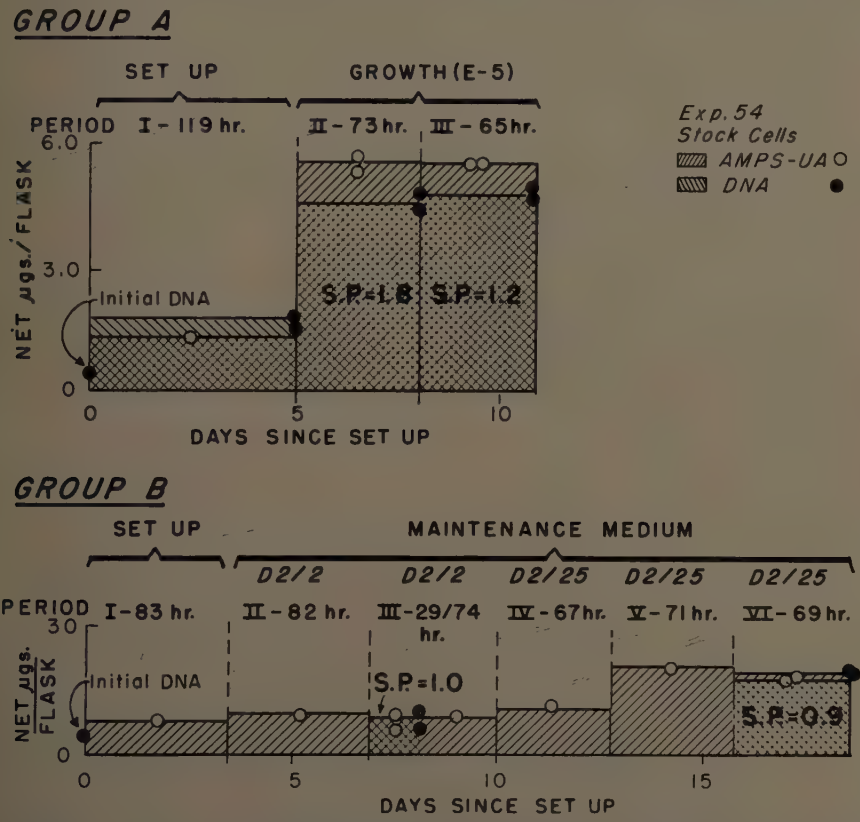

FIGURE 3. Similar to the experiment of FIGURE 2, except that a 3-year-old strain of rat fibroblasts was used. Flasks were separated at random into series A and B at 83 hours after setting up. Slow growth and constant AMPS production occurred in group B. In group A, specific production increased, then decreased as growth stopped.

the same levels as for period III at the conclusion of 19 days of maintenance with very slow growth.

Flasks of group A incubated 119 hours before a first change of medium demonstrated increased DNA content amounting to at least 3.6 times initial DNA; a considerable part of this increase must have occurred after 83 hours. Specific production in this prolonged setup period (I) was 1.17. In period II these flasks received growth medium (E-5), and DNA content per flask further increased an average of 2.6 times its prior value, while AMPS production per flask increased 4.3 times. Specific production increased to 1.76.

Flasks maintained an additional 65-hour period (III) on the same medium showed very slight changes in flask DNA content or in amount of AMPS produced as compared with flasks harvested at the end of period II. Specific production decreased to 1.15, however, two thirds of the previous value, since mean DNA content over period III was 50 per cent higher than in period II. An additional period of incubation with medium of high growth potential, in flasks with high cell densities, thus resulted in coincident decreases in DNA synthesis and specific AMPS production. At the same time, cell-bound AMPS increased almost threefold from period II to period III, as shown by TABLE 3.

Results of these two experiments again suggest a direct relationship between growth rate and AMPS production. Decreased rate of growth on medium of decreased nutrient content was accompanied by immediate and substantial reduction in amount of specific AMPS production. Decreased growth rate due to crowding had a similar effect.

TABLE 3
CELL-BOUND AMPS (EXPERIMENT 54)
Net Micrograms Uronic Acid per Flask*

Group	Period			
	I	II	III	VI
A	0.2	0.35	0.95	—
B	—	—	0.15	0.5

* Values are averages of two analyses.

STUDIES OF SOME METABOLIC FACTORS AND THEIR EFFECT UPON AMPS PRODUCTION AND GROWTH

Glucose Concentration

Several experiments were carried out in which glucose concentration of the medium was increased and decreased from the standard 0.1 per cent. Results indicated that as much as a sevenfold increase (3.7 times standard concentration at feeding plus additional concentrated glucose after 24 hours of incubation) or a fourfold decrease in glucose concentration did not bring about significant differences in AMPS production, as compared with control cultures receiving 0.1 per cent glucose. See TABLE 4 for results of two experiments.

Variation in pH

The effect of varying the *p*H of the medium was investigated in a total of 5 experiments; in 3 of these experiments other factors were varied as well. In one, the effect of changing gas phase CO_2 concentration from 5 to 0.5 per cent on both growth and maintenance media was investigated. In this experiment and in at least 1 other it appeared that growth was blocked by the high *p*H resulting from decreased CO_2 tension in the gas phase; in no case did production of AMPS appear to be materially affected by varying *p*H in this manner. However, these experiments were not repeated in a satisfactory manner, since

the cells tended to become detached and to retract into clumps at the high *p*H. Studies of *p*H effects were not continued because of the lack of satisfactory methods for maintaining and measuring the *p*H at specified levels.

Eagle's Medium Concentration

Results of experiments 54 and 55 indicated that the maintenance media used had, in fact, supported a slow rate of growth in both fresh and stock rat cells. Production of AMPS by cells on such maintenance media was variable, but lower than on growth media. For a more precise determination of minimal nutrient requirements for least growth and to study the effect of growth limited by starvation on specific production of AMPS, cultures were transferred to a

TABLE 4

EFFECT OF CHANGES IN GLUCOSE CONCENTRATION ON AMPS PRODUCTION BY FRESH AND STOCK RAT CELLS RECEIVING MAINTENANCE MEDIUM

Analytical values are in units of micrograms per flask; values for T-15 flasks determined on at least 2 pooled flask contents.

Medium (and flasks)	Glucose concentration (%)	Average net Supernate AMPS-uronic acid per flask in second period	Average DNA per flask	Specific AMPS-UA per DNA; second period
Fresh Rat Cells:				
Maintenance (T-15)	0.1	0.95	0.72	1.32
	0.04	1.07	0.80	1.34
Stock Rat Cells:				
Maintenance (T-15)	0.1	0.92	5.16	0.39
	0.04	1.17	4.58	0.41
Maintenance (T-60)	0.1	5.86	24.20	0.24
	0.37	5.21	26.36	0.20

Moderate variations in glucose concentration did not affect AMPS production. Systematic variation in *p*H was likewise ineffective.

medium containing 2 per cent of dialyzed placental serum either alone or with various low concentrations of Eagle's medium for 2 feeding periods. Results of one experiment with *stock* rat cells are shown in FIGURE 4 and TABLE 5. AMPS production in all flasks decreased in the first period from production in the previous period on growth medium: the decrease in production averaged 45 per cent for all T-15 flasks and 33 per cent for T-60 flasks on 25 per cent Eagle's medium. In the second period, AMPS production again decreased 15 to 58 per cent below production in the first period: the amount of the second period decrease was greatest in flasks receiving highest concentrations of Eagle's medium.

Total net growth (flask DNA content after 2 periods compared with average initial flask DNA content) was approximately proportional to the logarithm of the concentration of Eagle's medium supplied. In the absence of Eagle's medium, average flask DNA at the end of 2 periods was essentially unchanged; in the flask receiving 25 per cent of Eagle's medium (highest concentration

tested), average flask DNA increased 113 per cent over initial values. Specific AMPS production dropped sharply and progressively with increasing concentrations of Eagle's medium from a value of 1.0 in its absence to 0.18 in the presence of 25 per cent of Eagle's medium.

A similar experiment (experiment 52) carried out with fresh rat cells resulted in no net growth over 2 successive periods of 47 and 79 hours at concentrations

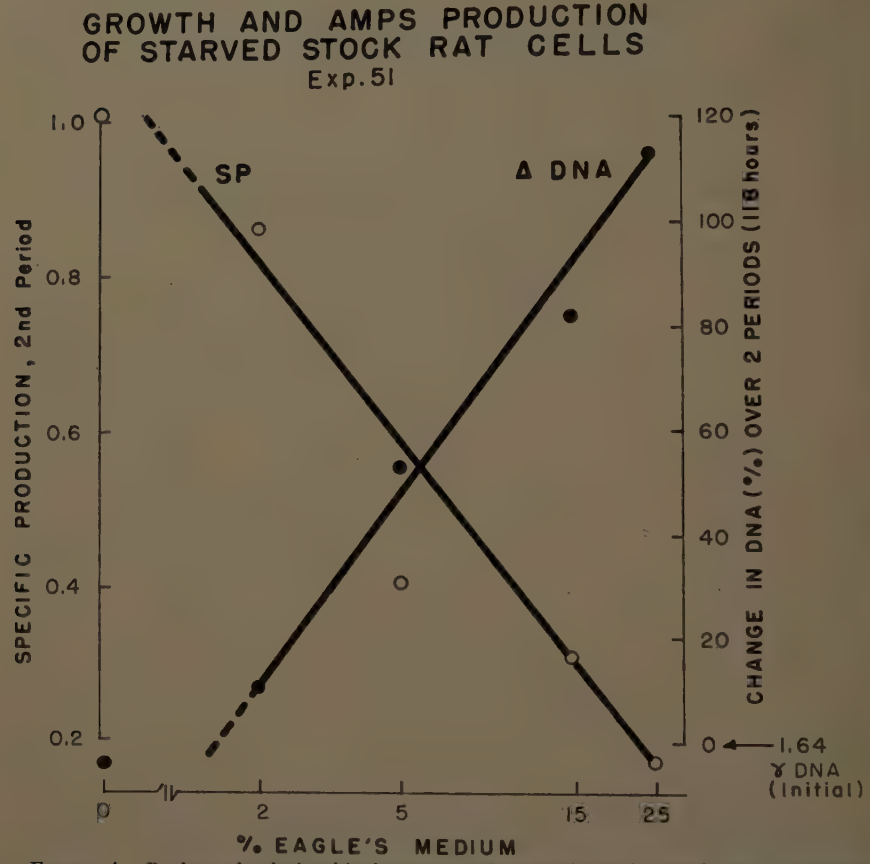

FIGURE 4. Reciprocal relationship between amino-acid-limited growth and production of AMPS by starved cells. Eagle's medium concentration is plotted logarithmically. See TABLE 5 for additional details.

of Eagle's medium of less than 10 per cent. Mitoses were observed, and slight net growth may have occurred with 10 per cent of Eagle's medium and glutamine at 10 μg./ml. In the absence of Eagle's medium the cells did not, in fact, survive 2 complete periods, but appeared to be attenuated or necrotic, and were almost all detached from the glass at the end of the second period. In the flasks receiving 2 per cent of Eagle's medium, a much lower proportion of cells was detached. However, specific production in all flasks during the second period remained at or above 1.1, except for flasks on 10 per cent Eagle's me-

dium plus glutamine, where it averaged 0.75; these are the flasks in which slight growth may have occurred. Such a correlation between decreased specific production and very slight growth parallels the findings in experiment 51; the fresh cells, however, were unable to survive or to grow in low concentrations of Eagle's medium, satisfactory for slow growth of the stock cells. The most starved and apparently dying cells, on the other hand, continued to produce substantial amounts of AMPS in the second period.

TABLE 5
GROWTH AND AMPS PRODUCTION OF STARVED STOCK RAT CELLS
(Experiment 51)

Medium-D2/-2% PS-D with Eagle's HeLa med. as indicated (%)	% change in DNA End of 2nd period. Initial DNA—1.64 μg	Specific AMPS production (2nd period)	Glutamine 10γ/ml.	Net supernate AMPS-uronic acid/flk.		Average DNA per flask
				1st per. 66 hr.	2nd per. 52 hr.	
0	−5	1.0 ± 0.08	−	2.33	1.52	1.38
			+	1.95	1.50	1.73
				(−16%)		
2	+11	0.86 ± 0.18	−	2.18	1.38	1.75
			+	1.95	1.51	1.89
				(−11%)		
5	+53	0.36 ± 0.06	−	2.28	1.21	3.02
			+	1.77	0.53? (1)	2.00?
				(−22%)		
15	+82	0.31 ± 0.01	−	2.20	0.93 (1)	3.12
			+	1.98	0.86	2.86
				(−10%)		
25 (T-60)	+113	0.18 (2)	−	10.61 (1)	4.83 (1)	23.3 (1)
			+	8.80 (1)	3.82 (1)	26.5 (1)
				(−17%)		

Data of experiment of FIGURE 4. Initial DNA per T-60 flask was 11.7 micrograms; other values are in micrograms where not noted. Addition of glutamine to some flasks resulted in a decrease in AMPS production during the first period averaging 15%.

THE EFFECT OF GLUTAMINE UPON GROWTH AND AMPS PRODUCTION

Effect of Glutamine in Growth-Limiting Media

Preliminary tests of the effect of glutamine on AMPS production, where the glutamine was added, at concentrations of from 10 to 300 μg./ml., to standard growth and maintenance media, were inconclusive. Subsequent investigations of the effect of glutamine were carried out under altered conditions, involving either pretreated cells or deficient media. In experiment 51, where increasing low concentrations of Eagle's medium resulted in increasing growth but depressed AMPS production of starved stock rat cells, some flasks at each concentration of Eagle's medium were tested for the effect of 10 μg./ml. of added-glutamine (TABLE 5).

Glutamine did not affect AMPS production after a 24-hour incubation in the absence of Eagle's medium; specific AMPS production was 0.25 in this instance (this datum was not included in TABLE 5). The presence of glutamine resulted in a definite inhibiting effect on AMPS production of the remaining flasks. In the first feeding period, flasks receiving glutamine produced less than flasks without glutamine, in all 5 groups; the difference amounted to 10 to 22 per cent, with an average difference of 15 per cent, for the 5 groups. In the second experimental interval the individual values fluctuated more, and the differences were not significant, but appeared to exist in most instances. This inhibiting effect of glutamine upon AMPS production possibly can be correlated with an increased DNA content in the flasks of each group that received glutamine, although the data are not conclusive; in 3 of the 4 groups where DNA was determined unequivocally, average values for flasks receiving glutamine were higher than values for corresponding flasks without glutamine. Values for flask DNA content fluctuated widely in some instances, however, and the net effect attributable to glutamine is not statistically significant. In the similar experiment (experiment 52) with "fresh" rat cells, previously referred to, glutamine at 10 μg./ml. had no effect upon either growth or AMPS production, with one exception: flasks receiving 10 per cent Eagle's medium and glutamine showed a slight increase in DNA content and a 30 per cent decrease in specific production in each of 2 feeding periods over all other flasks less well nourished. Glutamine did promote minimal maintenance of the most starved cells, so that a higher proportion of them remained attached to the glass than in the absence of glutamine.

On Cells Preadapted to Excess or Deficiency of Glutamine

Glutamine was added at approximately 300 μg./ml. during several feeding periods in one experiment with stock rat cells, after which various lower concentrations of glutamine were supplied to different flasks for 2 additional periods. Results are summarized in TABLE 6. In this experiment all flasks produced and released to the medium approximately the same amounts of AMPS at each of 2 incubation periods; concentration of glutamine present did not appear to affect the amount of AMPS produced per flask. However, there was a marked effect of glutamine upon growth of the cells; in the absence of glutamine there was no increase in DNA content of the flasks over the 7-day period. Increasing concentrations of glutamine, to 100 μg./ml., resulted in increasing amounts of DNA synthesis. It therefore follows that specific production of AMPS decreased with increasing concentration of glutamine; specific AMPS production for the second and final feeding period ranged from a high of approximately 5.7 to a low of approximately 1.5, as glutamine concentration increased from 0 to 100 μg./ml.

After stock cells had been grown for some time in the absence of glutamine, a similar test of glutamine was carried out. It is apparent from the results (TABLE 7) that in this instance the amount of DNA synthesis was not affected by glutamine at any concentration tested over an 8-day period: all flasks showed a uniform increase in DNA content to a mean of 5.22 μg. per flask (S.E. of 8 means was 0.16 μg. per flask), which amounted to 2.2 times the initial

TABLE 6

CELLS PREADAPTED TO EXCESS GLUTAMINE

Effect of Glutamine Upon Growth and AMPS Production of Stock Rat Cells Following Preadaptation (Experiment 44).

Glutamine added μg./ml. (in DS2/98 medium)	Supernate AMPS uronic acid content net μg./flask		Cell-bound AMPS uronic acid content net μg./flask	DNA End of 2nd per. μg./flask		Specific production. Average, 2nd period. μg. Super UA per μg. DNA
	First period (3 days)	Second period (4 days)		Each pair	Average	
	(Pooled)			Initial: 1.28		
0	4.3	5.1 5.3	0.6 0.3	1.50 0.53	1.01	5.2
1	4.9	5.4	0.2	0.95	(0.95)	5.7
3	3.7	4.3 4.5	0.1 0.5	1.23 2.54	1.88	2.3
10	3.8	4.6 4.3	0.3 0.4	2.10 2.86	2.48	1.8
30	3.6	4.6 —	0.5 0.5	2.26 3.12	2.69	1.7
100	4.2	5.2 4.1	0.5 0.3	3.34 2.73	3.03	1.5

See text for details.

TABLE 7

CELLS PREADAPTED TO ABSENCE OF GLUTAMINE

Effect of Glutamine Upon Growth and AMPS Production of Stock Rat Cells Following Growth in its Absence (Experiment 47).

Glutamine added μg./ml. (in DS2/98 medium)	Supernate AMPS-uronic acid content net μg./flask			Cell-bound AMPS uronic acid content net μg./flask	DNA End of 3rd per. μg./flask	Specific production μg. super UA per μg. DNA	
	First period (2 d.)	Second period (3 d.)	Third period (3 d.)			2nd* per.	3rd per.
	Initial: {2.5 (T-15) / 9.4 (T-60)} (Pooled)		(after 4 d. on E-5)		{2.40 (T-15) / —}		
0	2.9	0.9	0.7 0.2	— 0.2	5.56 4.76	0.3	0.1 0.1
1	2.4	1.1	0.9 1.2	0.2 0.2	5.08 5.92	0.3	0.2 0.2
3 (T-60)	9.5	7.4	5.6	2.1	31.46	0.4	0.2
10	2.1	1.0	1.2 1.2	0.2 —	5.59 5.01	0.3	0.2 0.2
100	2.2	0.6	0.3 0.8	0.1 0.6	5.26 4.56	0.2	0.1 0.2

* Assumes exponential growth with mean 2nd period DNA approximately 62% of final DNA.

See text for details.

value. Production of soluble AMPS decreased with each successive feeding, almost without exception, from the average of 2.49 μg. of AMPS-UA per flask produced on growth medium in the interval just prior to the experimental feedings. Glutamine supplied had a questionable effect, in reversing this pattern of progressively decreasing production, at the 1 and the 10 μg./ml. levels. However, the T-60 flask supplied glutamine at 3 μg./ml. also showed the pattern of successive decreases in AMPS production with each feeding.

Specific production of AMPS in this experiment was low in all cases: it amounted to an average value of 1.04 at the beginning of the experimental period, and it decreased to an estimated average (assuming an exponential DNA increase over the 8 days) of approximately 0.32 at the end of the second experimental feeding period and to an average of 0.16 at the end of the third and final period.

Effect of Glutamine Following Preincubation

In one experiment (No. 57), flasks of stock cells were preincubated at 37° C. for 4 hours in maintenance medium without Eagle's components. Such treatment resulted in a small amount of AMPS production during the preincubation period followed by diminished and decreasing rate of AMPS production over 7 and 42 hours of further incubation following a change of medium. Total AMPS production over the 46 hours of preincubation plus incubation periods was 64 per cent of that for flasks incubated for 46 hours without a preincubation period. Glutamine at concentrations of 10, 100, and 400 μg./ml. had no effect.

When a similar experiment was carried out, but with flasks preincubated for 4 hours in the cold instead of at 37° C., subsequent production of soluble AMPS after 50 hours on maintenance medium averaged the same as production in the previous 96 hours on growth medium. In this experiment, S^{35}-sulfate was added to the preincubation and the incubation media, and radioactivity, as well as uronic acid content of the AMPS extracts, was determined.

Results of this experiment (No. 58) are given in TABLE 8. Production of soluble AMPS in the 7 to 50-hour interval fell off almost 50 per cent from the rate of the first 7 hours for flasks receiving no glutamine. In flasks receiving 150 μg./ml. of glutamine, the rate in the 7-to 50-hour interval fell off still more sharply: after 50 hours, the average difference (0.81 μg. of AMPS-UA) between the flasks with and those without glutamine is significant at close to the 1 per cent level; glutamine clearly inhibited net production of soluble AMPS, as determined by uronic acid content of the AMPS fraction.

The amount of radiosulfate fixed in soluble AMPS at 50 hours was 6 times the amount found at 7 hours; the 7- to 50-hour rate of sulfate fixation thus remained more nearly constant than net AMPS-UA production in this period, decreasing to approximately 80 per cent of the rate for the first 7 hours. In contrast to the 25 per cent inhibition of AMPS-UA in the presence of glutamine after 50 hours, there was no significant glutamine inhibition of radiosulfate fixation. At 7 hours there were no significant effects of glutamine on either uronic acid content of AMPS or in sulfate fixation. The data, while not conclusive, suggest that different factors control rates of AMPS production and rates of their sulfation by the fibroblast cell, although other possibilities cannot be excluded (see under **Discussion**).

In the 4 hours of prior preincubation at 3° C. a small amount of radiosulfate was incorporated into the soluble AMPS fraction along with a small net increase in soluble AMPS-UA. Two washes with cold unlabeled medium were sufficient quantitatively to remove loosely bound, labeled AMPS from the cells after preincubation. Preliminary investigation had established that labeled sulfate incubated with medium taken from the cultures did not exchange with soluble AMPS in the absence of cells.

Radiosulfate in the cell-bound AMPS fraction at 7 hours was approximately half of the amount present in the soluble AMPS fraction at that time. The content in the cell-bound fraction continued to increase somewhat, but its

TABLE 8

EFFECT OF GLUTAMINE ON AMPS PRODUCTION BY STOCK RAT CELLS (EXPERIMENT 58)

Parallel Determinations of S^{35}-Sulfate and Uronic Acid Content of AMPS Produced Flasks preincubated 4 hours at 2–3° C. Each value represents 2 or 3 (with SE).

Hours at 37° C.	D-2/0 medium Glutamine γ/ml.	Average net supernate AMPS		Average calculated ChS		Cell-bound AMPS Ave./set/flask	
		Uronic acid μg./flask	S^{35} cpm/flask	UA-γ	as % Total UA	Uronic acid	S^{35} cpm
7	0	0.7	556	.03	5.7	—	—
	30	0.75 ± .10	718 ± 41	.04	6.5	0.6	364
	150	0.83 ± .07	599 ± 35	.03	4.9	1.0	302
50	0	3.18 ± .19	3707 ± 71	.21	7.7	0.6	557
	150	2.37 ± .14	3542 ± 48	.20	9.8	0.5	559

See text for details. Assumed 'Chondroitin Sulfate' (ChS) was calculated from the radioactivity data, the specific activity of the medium sulfate, and the uronic acid values as indicated in the specific procedures sections under **Materials and Methods**. Values given are means of 3 determinations on 2 pooled flasks each where standard errors are given; otherwise, they are means of 2 determinations.

proportion decreased in relation to the soluble fraction so that it constituted only 15 per cent of the soluble fraction at 50 hours.

NATURE OF THE AMPS PRODUCED

The nature of the AMPS produced was indicated but not established by a variety of techniques in preliminary studies: first, partially purified AMPS extracted from pools of supernates were digested completely by both testicular and pneumococcal hyaluronidases, although bacterial hyaluronidases do not degrade the chondroitin sulfates; second, the dialyzable oligosaccharides after enzymatic digestion, when collected, concentrated and chromatographed, gave a pattern of spots indistinguishable from that obtained with the respective enzymatic digests of purified hyaluronic acid (method of Weissmann *et al.*, 1954); third, hydrolysis of the AMPS and chromatography of the hydrolysate (method

of Fischer and Nabel, 1955) indicated the presence of glucosamine but not galactosamine; fourth, calculating the specific activity of the S^{35}-sulfate in the incubation medium of experiment 58 made it possible to estimate the amount of presumed chondroitin sulfate present in the AMPS extracts and the fraction that this constituted of the total AMPS, estimated from uronic acid values. These estimates indicated that the "chondroitin sulfate" component was present as only 5 to 10 per cent of the total AMPS. This small fraction of chondroitin sulfate would not have been detected by the other methods. To the extent indicated by these limited studies, both the fresh cells and the stock cells growing for 3 years in culture produced a major fraction of hyaluronic acid and a minor fraction of a presumed chondroitin sulfate.

Morphologic and Histochemical Studies

The appearance of fibroblasts growing in culture has been described and pictured many times (see Cameron, 1952 for review; Parker, 1933; Fawcett, 1954; and Willmer, 1958 for figures). Appearance of the cells in this study in both primary and suspension cultures differed in no essential way from previous descriptions of similar material; they did not resemble the endosteal cells of bone matrix.

Definitive metachromasia of cells from suspension cultures was not observed. The colloidal iron technique gave a pattern of cellular staining that corresponded closely to the pattern of basophilia obtained with either azure B or toluidine blue.

The standard paS stain demonstrated (1) fairly uniform small cytoplasmic granules in a variable proportion of cells, and often in groups of cells in close proximity; (2) occasional larger amorphous irregular cell inclusions with distinct boundaries or similar material closely associated with cells; and (3) variable general or diffuse staining of cellular material, especially when cells were aggregated in strands or clusters several cells thick. Cytoplasmic granules of a distribution and appearance similar to those demonstrated with the paS stain were seen also in cells fixed and observed unstained under phase contrast. There was no apparent difference in the numbers of paS-positive granules under conditions where a difference of two and one-half times in the amount of cell-bound AMPS was found analytically.

Extraction with hot acetone or methanol-chloroform mixtures did not appear to diminish either granular, amorphous, or diffuse paS staining. No staining occurred when the periodate oxidation step was omitted from the procedure. Digestion with hyaluronidase for 30 min. had no effect upon the paS staining of either cytoplasmic granules or material in clumped cells. Treatment with either intact or boiled hyaluronidase for 4½ hours sharply diminished background paS staining, but paS staining of debris in cell clumps and of cytoplasmic granules was not affected appreciably. Digestion with saliva did not materially affect any of the 3 types of paS staining described.

Discussion

Range of AMPS Production per Cell

The only other work on AMPS production by a defined line of cells of connective tissue origin dispersed as a suspension is that of Castor (1959),

who compared hyaluronic acid production in cultures of human synovial cells over successive periods and also compared production by cells obtained from different persons.

Castor presented his data, obtained on the basis of cell counts made in the process of setting up new subcultures, in the form of the amount of AMPS produced per cell per day; this value ranged from 30×10^{-12} to 262×10^{-12} gm. under his standard conditions. Similar computations may be made for the amount and rate of AMPS production by cells in the present system, with the use of Leslie's figures (1955) for DNA content of the (predominantly) diploid rat cell. In the course of the present studies, some parallel counts of numbers of cells per milliliter of suspension and determinations of initial (cell-suspension aliquot) DNA were made to check replication. In 2 instances the DNA determinations were made on a sufficient number of samples to give a standard error, and an estimate of DNA per cell in suspension could be made, as shown in TABLE 9.

From this table it appears that Leslie's average value of 6.8×10^{-12} gm. DNA per cell (converted from DNA-phosphorus values) is a reasonable estimate to use. Taking uronic acid content to constitute approximately 40

TABLE 9

Expt. no.	Type cells	Per 2-ml. aliquot		No. DNA samples	Estimated DNA per cell ($\times 10^{-12}$ gm.)
		No. cells	DNA		
45	Fresh	77,000	0.44 ± 0.029	9	6.0
54	Stock	67,000	0.48 ± 0.013	5	7.1

per cent of the acid mucopolysaccharide molecule and Leslie's figure for cell DNA, the data of experiment 50 work out approximately as follows: specific production in periods II (3.1) and III (2.5) represents production of approximately 53×10^{-12} and 41×10^{-12} gm. of AMPS per average cell, respectively; and initial rates of production amount to 1.6×10^{-12} and 1.1×10^{-12} gm. of AMPS per average cell per hour, in the 2 successive periods.

Relation of Growth Rate and AMPS Production

If the data for the early intervals of periods II and III of experiment 50 are compared (FIGURE 1, TABLE 2), it is apparent that the rate of AMPS production is 40 to 50 per cent higher in the first 20 hours of period II than in the first 12 or 34 hours of period III. These different initial rates of AMPS production coincided with different degrees of mitotic activity, so that flask DNA content increased 126 per cent and 32 per cent in periods II and III, respectively. This correlation, also observed in other experiments, suggests a direct over-all relationship between rate of growth and rate of AMPS production by cells; the data of experiments 54 and 55 tend to confirm this relationship. Since in these last 2 experiments cellular AMPS production is defined in terms of a specific production, the justification for defining and using this term as it has been employed will be considered.

Specific Production of AMPS

This ratio has been defined for these studies in terms of an intermediate DNA, where flask DNA content changes during a feeding period. Selection of the intermediate DNA value was based upon the following considerations: use of either initial or final DNA for the calculation would be misleading; an increment of DNA that occurred late in a feeding period, representing new daughter cells, would not synthesize AMPS for as long a time nor as effectively (because of progressive medium depletion) as would DNA representing cells present from the beginning of the period. However, additional AMPS is produced late in at least some feeding periods after extensive growth. The additional AMPS in these instances could have been contributed only by new daughter cells after mitosis. An alternative hypothesis, that the original and the daughter cells are not equally competent with respect to intrinsic growth rate and AMPS production, is inadmissible, because this would have led to an inverse relation between total growth and specific production, which was never observed.

The use of mean period DNA appears to describe the situation quite accurately, at least for the conditions obtaining in periods II and III of experiment 50. In both of these periods, the point on the DNA curves representing the mean DNA value for the period corresponds closely in time with the point at which half the total AMPS to be produced in that period had already appeared. The analytic points are too few to establish this relationship with precision, but it seems permissible to interpret this correspondence as indicating that half the AMPS were produced in the interval during which half of the total DNA increase was taking place.

Growth and Specific Production Influenced by Medium and Crowding

In experiment 55 (FIGURE 2) the relationship between specific production and growth rate is direct and obvious (compare values for groups A and B in period III). However, the fact that decreased rate of growth and decreased production of AMPS occur together, when cells are transferred to maintenance medium, does not establish a direct relationship. Another type of growth limitation occurred in experiment 54 (FIGURE 3), where flasks in group A were continued on growth medium for 1 and 2 periods after the setup period. Flask population more than doubled (160 per cent increase) after the first feeding (period II), while specific production for the period increased approximately 50 per cent (to 1.76). After the second feeding (period III) on the same medium, the condition of crowding inhibited cell division almost completely, confirming recent quantitative studies made by others (for example, Harris, 1955*b*; Fioramonti *et al.*, 1958). With crowding, specific production decreased by more than one third (to 1.15).

AMPS production may well be reduced or stopped during the actual process of mitosis without greatly decreasing the amount of AMPS produced over an extended period, since the mitotic period, approximately 20 to 50 min. (Cameron, 1952), is only a small fraction of the cell generation time, approximately 15 to 40 hours, for rapidly growing rat fibroblasts (Harris,

1955*a*). AMPS production probably continues to within a short time of mitosis and commences in the daughter cells a short time after mitosis. The data of experiment 50 and other experiments indicated that rate of AMPS production was not decreased appreciably during periods when considerable DNA synthesis was occurring; Harris (1959) found that the median period of active DNA synthesis extended from 13 to 14 hours to 3 to 4 hours before mitosis in young rat heart fibroblasts.

Whatever the precise temporal relationship between AMPS production and mitosis, specific production in these experiments did not decrease except where growth rate was depressed: rate of AMPS synthesis remained highest in growing, that is, dividing, populations of cells, but fell somewhat when the cells were not actively proliferating. That the rate of AMPS synthesis is not decreased during intervals of active DNA synthesis suggests that these two processes need not be competitive.

Although growth rate and rate of production of AMPS were positively correlated in cells fed with growth medium, growth is not a necessary condition of production. Other data established the fact that cells dividing slowly or not at all produce large amounts of AMPS at quite constant rates over extended periods of time. Regardless of whether growth limitation was caused by crowding or by maintenance medium, specific production in growth-limited cultures reached a high of 1.0. Moreover, sequential analyses of supernates from extremely dense, nongrowing stock culture flasks, maintained without disturbance for many months to provide material for subcultures, indicated that average specific production remains quite constant at about 0.6; the syrupy supernate from such flasks invariably produced a heavy mucin clot.

"Competitive" Interaction of Slow Growth and AMPS Production

Cells on dilute maintenance medium adequate only for very slow growth rates produced less AMPS than did cells on maintenance medium too dilute to sustain growth. This is probably a competitive effect between growth processes and processes involved in AMPS production; limiting concentrations of substrates or products of the substrates may be involved. Since the same effect was observed in other experiments where vitamins and cofactor concentrations were held constant and only amino acid concentrations were changed, it is probably attributable to limiting concentrations of one or more amino acids among the 12 in Eagle's medium (FIGURE 4, TABLE 5).

Inhibition of AMPS production was observed also when similar cultures of human fibroblasts derived from adult temporal bone were permitted to grow slowly in the presence of low concentrations of Eagle's medium; this was not the case with nongrowing cultures without Eagle's medium (Morris, unpublished observations). In both the human and the rat cell, this effect was independent of the presence or absence of glutamine: in the rat cell, glutamine was ineffective or it depressed AMPS production somewhat at the same time while, in the human cell, glutamine stimulated AMPS production slightly, in contrast to controls without glutamine, whether or not Eagle's medium was present.

Results of Present Studies Varying Glucose Concentration and pH

Results of several experiments in the present study indicated that rate and amount of AMPS production by cultured rat fibroblast cells were quite insensitive to moderate variations in glucose levels and *p*H which, considering their relevance to the assumed metabolic pathways from glucose to the AMPS or to glucose metabolism in general, or to both, might have been expected to affect it. Such studies might be more profitably extended in strictly regulated systems, analogous to the complex "cytogenerator" of Graff and McCarty (1958), where glucose concentration and *p*H may be controlled, maintained, and determined over long periods of *in vitro* growth of animal cells. More drastic changes in these variables, or the use of metabolic poisons or inhibitors or other agents, might have brought about significant changes in the production of AMPS, but this study was arbitrarily restricted to experimental variables compatible with long-term maintenance, "normal" growth of cells in culture, or both. The present studies may serve as background for future work with hormones and other agents known to have profound effects upon connective tissue metabolism (Dorfman and Mathews, 1956; Asboe-Hansen, 1958, 1959).

Lack of Direct Stimulatory Effect of Glutamine on AMPS Production in These Studies

The role of glutamine in connective tissue AMPS production is somewhat controversial; while glutamine has been identified as active in some preparations synthesizing glucosamine-6-phosphate from glucose- or fructose-phosphate (Leloir and Cardini, 1953; Pogell and Gryder, 1957; Castellani and Zambotti, 1958), in other systems (Leloir and Cardini, 1956; Comb and Roseman, 1956) ammonia is the source of the amide-nitrogen. In the present study, glutamine either was ineffective or slightly inhibited AMPS production when added to a medium supporting rat cell growth or maintenance. Furthermore, exogenous glutamine was not required for maintenance of the cultured rat fibroblast, not even by "stock" strains with long *in vitro* history and, except in the special case of subcultures preadapted to an excess, glutamine was not demonstrably effective in promoting cell growth.

Complete absence of added glutamine (TABLE 7) brought about marginal changes in metabolism. In contrast, cells preadapted to excess glutamine (TABLE 6) showed a marked dependence upon glutamine for growth but, since production of AMPS remained practically constant for all flasks, specific production decreased inversely with growth. Such an effect of glutamine upon growth was not found during any other experiment. Dependence upon glutamine for growth in culture was found by others to be characteristic of established and neoplastic cell strains, but not for several normal cells (Pasieka and Morgan, 1959). Together with the uniquely high values for specific production encountered in this experiment, the data suggest that changes in metabolic patterns of the cells have occurred as a result of their preadaptation to glutamine, perhaps by a mechanism similar to that noted by DeMars in 1958 (see below). However, specific production of AMPS by the cells in the preadaptation experiment reached its highest value (5.7), one of the highest found in any experiment, without added glutamine.

Possible Mechanisms of Observed Glutamine Effects

Although one laboratory (Bostrom *et al.*, 1955; Roden, 1956*a*, *b*, *c*) has reported that glutamine stimulates fixation of S^{35}-sulfate and of C^{14}-labeled glucose into sulfated mucopolysaccharide of calf cartilage and pig nucleus pulposus slices, the net synthesis of AMPS was not studied in that instance, and there have been no reports of glutamine stimulation of net AMPS synthesis in connective tissue systems. In a recent study (Pogell, 1959) glutamine stimulated the labeling of tissue hexosamines two- or threefold when bovine corneas were incubated *in vitro* in the presence of C^{14}-labeled glucose; however, it was not established that this represented either AMPS hexosamine or net synthesis, and the very low glucose and high glutamine concentrations used make interpretation uncertain. Other reservations have been expressed regarding the cell-free system of Castellani and Zambotti (1958), and its recent extensions (Paronetto, 1959).

A recent report concerning the effects of medium glutamine on glutamine metabolism of cultured HeLa cells suggests a mechanism for the metabolic changes observed in cells preadapted to excess glutamine (experiment 44). DeMars (1958) showed that both the specific activities of glutamyl transferase and those of glutamyl synthase enzymes (which may be different activities of the same enzyme) were greatly depleted when HeLa cells were grown in high cencentrations of glutamine. These activities were reversibly restored fifteenfold to high levels when the cells were grown in the presence of high concentrations of glutamic acid without glutamine. These two enzymatic activities together constitute key pathways for glutamine synthesis and its utilization in cellular metabolism; a similar inhibition of these activities brought about by adapting cells in the present study to concentrations of glutamine slightly higher than those used by DeMars, would account for their inability to grow subsequently without it.

The reasons for the unusually high specific production of AMPS by those preadapted cells that subsequently received no exogenous glutamine are not immediately apparent. The possibility remains that connective tissue cells are able to form hexosamine intermediates for AMPS synthesis from glucose, using ammonia or other nitrogen sources besides glutamine, as suggested by Leloir and Cardini (1956) for pig kidney extracts. The data support this possibility; on the other hand, if endogenous glutamine is involved in AMPS production in these cells independently of exogenous glutamine, then questions must be raised regarding the nature of such endogenous "glutamine" and its equilibrium with other intracellular pools of glutamine. Exogenous glutamine appears to be used by many other cells and tissues essentially in the form supplied them, via known steps in cellular glutamine metabolism (Levintow *et al.* 1957; McCarty and Graff, 1959). Furthermore, a direct effect of glutamine on growth was observed under special conditions in the present studies. It would be of interest to observe whether ammonium salts have any effect upon AMPS production in this or similar systems: it is conceivable that a mechanism such as that indicated by DeMars had made available excess unbound ammonia for AMPS synthesis to those nongrowing cells subsequently deprived of glutamine in the preadaptation experiment. If formation of hexosamine intermediates directly from glucose and ammonia were involved, it would explain the

unusually high specific production of glutamine-preadapted cells in the absence of added glutamine.

Depression of AMPS Synthesis in the Presence of Glutamine

The results of three experiments indicated a degree of inhibition of specific AMPS production in the presence of added glutamine at both low and moderately high concentrations. In all cases, the cells had just been transferred to a deficient maintenance medium after a prolonged feeding interval on growth medium that included standard concentrations of glutamine. In experiment 58 (TABLE 8) the cells were additionally depleted before the test period by preincubation at ice-bath temperature with deficient medium. As adjudged from the results of experiments 51 (TABLE 5) and 52, it appears likely that glutamine had shunted metabolic efforts of the depleted cells toward maintenance and growth at the expense of AMPS production; however, the data are not conclusive. A similar glutamine stimulation of marginal growth on deficient medium might explain the inhibition of AMPS production observed in the presence of glutamine in experiment 58. Since glutamine is a key intermediate for utilization of other metabolites in growth and maintenance of many cultured cells, the over-all mechanism in these instances is probably similar to the one involved in the competitive aspects of growth and AMPS production with limiting amino acid concentrations.

Radiosulfate Study: Independence of AMPS Synthesis and Sulfation

The present data suggest that AMPS production and sulfate fixation proceed at different rates. That sulfation of the AMPS molecule might proceed independently of its synthesis and polymerization was suggested by Meyer in interpreting results of studies of AMPS fractions from tissue cultures of embryonic bones which indicated that the sulfated components might be "undersulfated" (Grossfeld *et al.*, 1957). This appears to be confirmed by the studies of D'Abramo and Lipmann (1957), of Suzuki and Strominger (1959), and of Korn (1959), in which "active sulfate," synthesized in cell-free systems, is attached by ester linkage to sulfated mucopolysaccharides when present, but otherwise to other acceptors. Because glutamine was not effective in promoting AMPS synthesis in the present study, it would not be expected to increase the amount of sulfate fixation in AMPS.

Binding of AMPS to the Cells: Significance of the Present Studies

Most kinds of AMPS can be readily extracted from connective tissues, either alone or complexed with specific proteins (Meyer, 1953, 1959; Malawista and Schubert, 1958; Muir, 1958; Partridge and Davis, 1958; Ogston and Sherman, 1959). A single layer of dissociated cells or of cells reassociated in a thin sheet with scant formed ground substance might be expected to bind AMPS less firmly than does the usually abundant ground substance of formed tissue. This is particularly true when the AMPS is chiefly of the hyaluronic acid type, as in the present study. It is well known that hyaluronic acid is very hydrophilic and is bound loosely, if at all, to protein. Two other probable factors

are (1) the presence of a large excess of aqueous medium bathing the cells in culture, rather than the restricted diffusion of tissue water (Edelman, 1952; Edelman and Leibman, 1959), and (2) the possibility of altered cell metabolism in culture, resulting in production of less ground substance protein for binding the AMPS.

Soluble AMPS appeared with little or no delay after a change of medium, as indicated in experiment 50 and in additional studies of AMPS released within four to five and one-half hours of a medium change. The rapid appearance of this soluble AMPS was not simply an extraction of previously formed cell-bound material, since the amount of cell-bound AMPS also increased soon after a change of medium.

In several instances in which growth stopped or continued at a very low rate for extended periods, cell-bound AMPS tended to accumulate. If, as an index of the amount of AMPS bound per cell, the amount of cell-bound AMPS-UA found is divided by the amount of DNA present, some comparative results may be presented (TABLE 10). A similar increase in cell-bound AMPS was

TABLE 10

Expt.	Group	Period	Increase in DNA over previous period (%)	Cell-bound AMPS-UA/DNA ratio	Change in (%)	Cause of decreased growth
	A	II	165	0.08	+150	Crowding
54 (FIG. 3)	A	III	5	0.20		
	B	VI	(Est. 32)	0.26	+30	Reduced nutrition
	A	III	210	0.15	+155	Reduced nutrition
55 (FIG. 2)	B	III	0	0.38		

observed in extremely crowded, static Carrel flask cultures of stock cells maintained on A-3 medium, from which material was taken to set up subcultures used in preparing cell suspensions. In these cultures, cell population density frequently was more than twice that of the most crowded suspension cultures. In 2 such cultures selected at random and analyzed, the cell-bound AMPS-UA/DNA ratios were 1.08 and 0.75, respectively; the fraction bound to the cells in these instances averaged 1½ times the very large amount of AMPS found in pooled supernates collected from similar cultures.

The correlation between increased binding of AMPS to tissues and decreased rate of division of constituent cells might have at least two explanations: a concomitant increase in production or retention of ground substance protein or fibrous protein with cessation of active "growth", or "squeezing out" of their AMPS content by dividing cells, possibly as a consequence of the mitotic act. The small amounts of cell-bound AMPS frequently resulted in erratic values in the conventional (uronic acid) method of estimation. The study with radiosulfate (TABLE 8), however, appeared to confirm that only a small part of the AMPS (sulfated in this case) was bound to cells where rate of production was high.

Significance of the Histochemical Studies

The essentially negative findings of these studies are consistent with a great deal of previous work by others, which has established that hyaluronic acid is not metachromatic at low concentrations (Sylven and Malmgren, 1952; Walton and Ricketts, 1954; Windrum, 1958) and that the paS reaction does not stain connective tissue AMPS, although it was used previously for this purpose (Greulich and Friberg, 1957; Lever and Ford, 1958; Meyer, 1957). The numbers of paS-positive granules present in the cytoplasm (Jackson, 1955) did not appear to be correlated with the rate of AMPS production; the presence of such granules, therefore, cannot readily be invoked as representing segregated precursor substances or sites for AMPS production.

Cell Differentiation and the Nature of the AMPS Produced

The finding that these cultured cells produce very small amounts of sulfated AMPS seems noteworthy: tissue culture studies of AMPS produced by cells growing from embryonic beef bone fragments (Grossfeld *et al.*, 1957) are in accord both with studies of the AMPS in early fracture callus *in vivo* (Maurer and Hudack, 1952) and with the present studies. In all cases hyaluronic acid appeared to predominate, while sulfated components (chondroitin sulfate C in the 1957 tissue culture study) were minor. This is in sharp contrast to studies of calf and beef bone AMPS content (Meyer, 1956; Meyer *et al.*, 1956), where hyaluronic acid is a minor component or is absent. Such findings support the view that growing fibroblasts in tissue culture revert to a "primitive" type of function (Willmer, 1958) analogous to that of fibroblasts of early embryonic or early wound tissue.

Comparison of Stock and Fresh Cells

The fresh and the stock cells appeared not to differ significantly in the amount or kinds of AMPS they were producing, although they differed slightly in their nutritional requirements and rate of growth. Prolonged periods of *in vitro* culture frequently result in permanent changes in cells; these changes include aneuploidy, changed DNA complement, and changes in growth rate, nutritional requirements, antigenic properties, enzyme activities, and tumor-inducing capacities, if reimplanted into an animal (Grobstein, 1959, pp. 477 ff.; Rinaldini, 1958). In addition, longer cultures of human synovium have been reported to lose their capacity to produce AMPS (Kling *et al.*, 1955; Castor, 1957, 1959) or to have it impaired. The present finding of marginal differences only between recently isolated cells and an established cell strain is noteworthy, considering the numerous examples of cell "alterations" in tissue culture, where such cultures are not maintained under "optimal" conditions (Tjio and Puck, 1958). If definitive tissue differentiation is controlled by factors manifested only in aggregates of cells, as is held by many embryologists (Grobstein, 1959), the amount and rate of uncontrolled cellular growth occurring during the first weeks of *in vitro* culture might well be accompanied by reversion to a primitive pattern of AMPS production. Additional periods of adequate maintenance and growth in the neutral milieu of tissue culture might be expected to elicit

no further changes (except for occasional random neoplastic changes) in the now adapted and stabilized tissue culture fibroblast strain.

Summary

Osteogenic rat fibroblasts produce and release considerable quantities of acid mucopolysaccharides (AMPS) into the culture medium when grown as a single cell layer on glass in T-15 flasks. A much smaller amount of AMPS is retained by the rapidly growing cells; this amount increases when growth is slowed or stopped. By the use of replicate cultures prepared from cell suspensions, growth rate was estimated for these studies as increase in average flask DNA content. AMPS was extracted from the used medium and cells and the uronic acid content of the extract was estimated; AMPS production was expressed as "specific production" (per mean microgram of DNA per period) or as a "rate constant" (specific production per hour). Simultaneous cell counts indicated that maximal AMPS production ranged from about 40 to about 50 $\mu\mu$g. of AMPS per cell per feeding period, and initial rates of 1.1 and 1.6 $\mu\mu$g. of AMPS per cell per hour were observed.

Growth and production of AMPS were compared for cells recently isolated from calvaria of young rats and for cells of similar origin maintained for three years in culture. In both types of cells, the relationship between growth rate and production of AMPS was investigated as a function of medium composition, "crowding" of cultures, and *p*H and concentration of glucose and glutamine in the medium. Slight differences in growth rate and behavior between the recently isolated cells and the stock cells were noted; there was no apparent difference between them in production of AMPS. Moderate variations in *p*H and glucose concentration did not affect AMPS production.

Rapid growth was accompanied by higher rates of specific AMPS production than occurred with slower growth rates or in the absence of growth; AMPS production remained at moderately high and constant levels during extended periods of slow growth. Production dropped to lowest levels and appeared to be competitive with growth when growth was limited by the concentration of Eagle's amino acid mixture present.

Glutamine did not directly stimulate AMPS production in well-nourished cells, starved cells, or cells rapidly depleted of endogenous reserves during a preincubation period. Glutamine, in fact, inhibited AMPS production somewhat in starved and depleted cells, possibly by participating in competitive metabolic activities essential for growth. These cells normally did not require glutamine for growth or maintenance. If preadapted to excess glutamine, glutamine was then essential for growth; the specific production of AMPS was inversely proportional to growth under these conditions.

Preliminary chemical studies indicated that both types of cells produce chiefly a nonsulfated AMPS, apparently hyaluronic acid. A study with S^{35}-sulfate indicated also that 5 to 10 per cent of a sulfated component was produced by the stock cells. This component was not identified further. Histochemical and cytological observations of living and fixed cells taken from flasks of known AMPS content failed to show any differences in appearance or staining reactions between cells with different amounts of AMPS produced by, or bound to, them.

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References

Asboe-Hansen, G. 1958. Hormonal effects on connective tissue. Physiol. Rev. **38:** 446–462.

Asboe-Hansen, G. 1959. Endocrine control of connective tissue. Am. J. Med. **26:** 470–484.

Becker, C. E. & H. G. Day. 1953. Utilization of glucosone and the synthesis of glucosamine in the rat. J. Biol. Chem. **201:** 795–801.

Berenson, G. S., W. M. Lumpkin & V. O. Shipp. 1958. Study of the time-course production of acid mucopolysaccharides by fibroblasts in a synthetic medium. Anat. Record. **132:** 585–596.

Boas, N. F. 1953. Method for the determination of hexosamines in tissues. J. Biol. Chem. **204:** 553–561.

Bollet, A. J. 1958. The measurement of tissue acid mucopolysaccharides. J. Clin. Invest. **37:** 858–863.

Bollett, A. J., M. W. Seraydarian & W. F. Simpson. 1957. Acid mucopolysaccharides in normal serum. J. Clin. Invest. **36:** 1328–1332.

Bostrom, H., L. Roden & A. Vestermark. 1955. Glutamine as an accelerator of chondroitin sulfate synthesis. Nature. **176:** 601.

Burton, K. 1956. A study of the conditions and mechanisms of the diphenylamine reaction for the colorimetric estimation of DNA. Biochem. J. **62:** 315–3231.

Cameron, G. R. 1952. Pathology of the Cell. Oliver and Boyd. London, England. : 103, 452.

Castellani, A. A. & V. Zambotti. 1958. Enzymatic formation of hexosamine in epiphyseal cartilage homogenate. Nature. **178:** 313.

Castor, C. W. 1957. Production of mucopolysaccharides by synovial cells in a simplified tissue culture medium. Proc. Soc. Exptl. Biol. **94:** 51–56.

Castor, C. W. 1959. The rate of hyaluronic acid production by human synovial cells studied in tissue culture. Arthritis and Rheumatism. **2:** 259–265.

Comb, D. G. & S. Roseman. 1956. Glucosamine-6-phosphate deaminase. Biochim. Biophys. Acta. **21:** 193–194.

D'Abramo, F. & F. Lipmann. 1957. The formation of adenosine-3′-phosphate-5′-phosphosulfate in extracts of chick embryo cartilage and its conversion into chondroitin sulfate. Biochim. Biophys. Acta. **225:** 211–213.

Deitch, A. D. & G. C. Godman. 1955. The application of a freezing-substitution method of fixation to tissue culture preparations. Anat. Record. **123:** 1–18.

DeMars, R. 1958. The inhibition by glutamine of glutamyl transferase formation in cultures of human cells. Biochim. Biophys. Acta. **27:** 435–436.

Dische, Z. 1947. A new specific color reaction of hexuronic acids. J. Biol. Chem. **167:** 189–198.

Dorfman, A. & M. B. Mathews. 1956. The physiology of connective tissue. Ann. Rev. Physiol. **18:** 69–88.

Edelman, I. S. 1952. Exchange of water between blood and tissues. Characteristics of deuterium oxide equilibration in body water. Am. J. Physiol. **171:** 279–296.

Edelman, I. S. & J. Leibman. 1959. Anatomy of body water and electrolytes. Am. J. Med. **27:** 256–277.

Evans, V. J., W. R. Earle, K. K. Sanford, J. E. Shannon & H. K. Waltz. 1951. The preparation and handling of replicate tissue cultures for quantitative studies. J. Natl. Cancer Inst. **11:** 907–927.

Evans, V. J., J. C. Bryant, W. T. McQuirkin, M. C. Fioramonti, K. K. Sanford, B. B. Westfall & W. R. Earle. 1957. Studies of nutrient media for tissue culture cells in vitro. III. Whole egg ultrafiltrate for long-term cultivation of strain L cells. Cancer Research. **17:** 317–324.

Fawcett, D. W. 1954. Mesenchyma and connective tissue. Chapt. 6. *In* Histology. R. O. Greep, Ed. Blakiston. New York, N. Y.

Fioramonti, M. C., V. J. Evans & W. R. Earle. 1958. The effect of innoculum size on proliferation of NCTC-L/929/2071 on chemically defined media. J. Natl. Cancer Inst. **21:** 579–583.

Fischer, F. G. & H. J. Nabel. 1955. Nachweis und Bestimmung von Glucosamin und Galactosamin auf Papierchromatogrammen. Z. Physiol. Chem. Hoppe-Seyler's. **302:** 10–19.

Flax, M. & M. Himes. 1952. Microspectrophotometric analysis of metachromatic staining of nucleic acids. Physiol. Z. **25:** 297–311.

Graff, S. & K. McCarty. 1958. Energy costs of growth in continuous metazoan cell culture. Cancer Research. **18:** 741–746.

Greulich, R. C. & N. Friberg. 1957. Histochemical studies of sulfomucopolysaccharides in the organic matrices of mineralized tissues. Exptl. Cell Research. **12:** 685–689.

Grobstein, C. 1959. Differentiation of vertebrate cells. *In* The Cell. Brachet and Mirsky, Eds. **I:** 437–496. Academic Press. New York, N. Y.

Grossfeld, H., K. Meyer & G. Godman. 1955. Differentiation of fibroblasts in tissue culture, as determined by mucopolysaccharide production. Proc. Soc. Exptl. Biol. **88:** 31–35.

Grossfeld, H., K. Meyer, G. Godman & A. Linker. 1957. Mucopolysaccharides produced in tissue culture. J. Biophys. Biochem. Cytol. **3:** 391–396.

Hallén, A. 1958. Hexosamine and ester sulfate content of the human nucleus pulposus at different ages. Acta Chem. Scand. **12:** 1869–1872.

Harris, H. 1955*a*. Some quantitative studies on the multiplication of connective tissue cells *in vitro*. Brit. J. Exptl. Pathol. **36:** 115–127.

Harris, H. 1955*b*. The sulphur requirement of rat connective tissue cells. Brit. J. Exptl. Pathol. **36:** 454–459.

Harris, H. 1959. The initiation of DNA synthesis in the connective tissue cell, with some observations on the function of the nucleolus. Biochem. J. **72:** 54–60.

Healy, G. M., D. C. Fisher & R. C. Parker. 1954. Nutrition of animal cells in tissue culture. VIII. Desoxyribonucleic acid phosphorus as a measure of cell multiplication in replicate cultures. Can. J. Biochem. **32:** 319–326.

Hedberg, H. & U. Moritz. 1958. Biosynthesis of hyaluronic acid in tissue cultures of human synovial membrane. Proc. Soc. Exptl. Biol. **98:** 80–84.

Hoffman, P., A. Linker & K. Meyer. 1957. The acid mucopolysaccharides of connective tissues. II. Further experiments on chondroitin sulfate B. Arch. Biochem. **69:** 435–440.

Jackson, D. S. 1957. Connective tissue growth stimulated by carrageenin. I. The formation and removal of collagen. Biochem. J. **65:** 277–284.

Jackson, S. F. 1955. Cytoplasmic granules in fibrogenic cells. Nature. **175:** 39–40.

Kaplan, D. & K. Meyer. 1959. Ageing of human cartilage. Nature. **183:** 1267.

Kling, D. H., M. G. Levine & S. Wise. 1955. Mucopolysaccharides in tissue cultures of human and mammalian synovial membrane. Proc. Soc. Exptl. Biol. **89:** 261–263.

Korn, E. D. 1959. The enzymatic sulfation of heparin. J. Biol. Chem. **235:** 1647–1650.

Kramer, H. & G. M. Windrum. 1955. The metachromatic staining reaction. J. Histol. Cytochem. **3:** 227–237.

Leloir, L. F. & C. E. Cardini. 1953. Enzymes acting on glucosamine phosphates. Biochem. Biophys. Acta. **12:** 15–22.

Leloir, L. F. & C. E. Cardini. 1956. Enzymes acting on glucosamine phosphates. Biochim. Biophys. Acta. **20:** 33–42.

Leslie, I. 1955. The nucleic acid content of tissues and cells. *In* The Nucleic Acids. II. Chargaff and Davidson, Eds. Academic Press. New York, N. Y.

Lever, J. D. & E. H. R. Ford. 1958. Histological, histochemical, and electron microscopic observations on synovial membrane. Anat. Record. **132:** 525–534.

Levintow, L., H. Eagle & K. A. Piez. 1957. The role of glutamine in protein biosynthesis in tissue culture. J. Biol. Chem. **227:** 929–941.

Loewi, G. 1953. Changes in the ground substance of ageing cartilage. J. Pathol. Bacteriol. **65:** 381–388.

Loewi, G. & K. Meyer. 1958. The acid mucopolysaccharides of embryonic skin. Biochim. Biophys. Acta. **27:** 453–456.

Lowther, D. A. & H. J. Rogers. 1956. The role of glutamine in the biosynthesis of hyaluronate by streptococcal suspensions. Biochem. J. **62:** 304–308.

Malawista, I. & M. Schubert. 1958. Chondromucoprotein; new extraction method and alkaline degradation. J. Biol. Chem. **230:** 535–544.

Maurer, P. & S. S. Hudack. 1952. The isolation of hyaluronic acid from callus tissue of early healing. Arch. Biochem. **38:** 49–53.

McCarty, K. S. & S. Graff. 1959. Some aspects of nitrogen metabolism in Strain L cultures. Exptl. Cell Research. **16:** 518–526.

Meyer, K. 1953. Hyaluronic acid, chondroitin sulfates and their protein complexes. Faraday Soc. Discussion **13:** 271–275.

Meyer, K. 1956. The mucopolysaccharides of bone. *In* Ciba Foundation Symposium on Bone Structure and Metabolism. : 65–73. Little, Brown. Boston, Mass.

Meyer, K. 1957. The chemistry of the mesodermal ground substances. Harvey Lecture Series LI (1955–1956). Academic Press. New York, N. Y.

Meyer, K. 1959. Chondroitin Sulfates (with others: see index). *In* Polysaccharides in Biology. Transactions of the Fourth Conference, 1958. G. Springer, Ed. Josiah Macy, Jr. Foundation. New York, N. Y.

Meyer, K., E. A. Davidson, A. A. Linker & P. Hoffman. 1956. The acid mucopolysaccharides of connective tissue. Biochim. Biophys. Acta. **21:** 506–518.

Meyer, K., P. Hoffman & A. Linker. 1958. Mucopolysaccharides of costal cartilage. Science. **128:** 896–897.

Muir, H. 1958. The nature of the link between protein and carbohydrate of a chondroitin sulfate complex from hyaline cartilage. Biochem. J. **69:** 195–204.

Noble, N. L. & R. J. Boucek. 1958. Relationship of glucosamine and galactosamine to collagen formation. Federation Proc. **17:** 399.

Ogston, A. G. & T. F. Sherman. 1959. Degradation of the hyaluronic acid complex of synovial fluid by proteolytic enzymes and by ethylenediaminetetra-acetic acid. Biochem. J. **72:** 301–304.

Parker, R. C. 1933. The races that constitute the group of common fibroblasts. II. The effect of blood serum. J. Exptl. Med. **58:** 97–113.

Paronetto, F. 1959. Interfering substances in the determination of glucosamine synthesis. Nature. **184:** 1317.

Partridge, S. M. & H. F. Davis. 1958. The presence in cartilage of a complex containing chondroitin sulfate combined with a non-collagenous protein. *In* Ciba Foundation Symposium on the Chemistry and Biology of Mucopolysaccharides. : 93–110. Little Brown. Boston, Mass.

Pasieka, A. E. & J. F. Morgan. 1959. Glutamine metabolism of normal and malignant cells cultivated in synthetic media. Nature. **183:** 1201.

Paul, J. 1959. Cell and Tissue Culture. Livingstone. Edinburgh, Scotland & London, England.

Pearse, A. G. E. 1953. Histochemistry: Theoretical and Applied. Little, Brown. New York, N. Y.

Pogell, B. M. 1959. Biosynthesis of mucopolysaccharides in bovine cornea. Biochim. Biophys. Acta. **31:** 280–283.

Pogell, B. M. & R. N. Gryder. 1957. Enzymatic synthesis of glucosamine-6-phosphate in rat liver. J. Biol. Chem. **228:** 701–712.

Rinaldini, L. M. J. 1958. The isolation of living cells from animal tissues. Intern. Rev. Cytol. **7:** 587–647.

Roden, L. 1956*a*. Effect of glutamine on the synthesis of chondroitin sulphuric acid *in vitro*. Arkiv. Kemi. **10:** 333–344.

Roden, L. 1956*b*. Isolation and identification of a liver factor stimulating the incorporation of S^{35}-labelled sulfate into chondroitin sulphuric acid *in vitro*. Arkiv Kemi. **10:** 325–332.

Roden, L. 1956*c*. Effect of glutamine on the synthesis of carbohydrates in the nucleus pulposus *in vitro*. Arkiv Kemi. **10:** 383–395.

Roseman, S., F. E. Moses, J. Ludowieg & A. Dorfman. 1953. The biosynthesis of hyaluronic acid by group A streptococcus. I. Utilization of 1-C^{14}-glucose. J. Biol. Chem. **203:** 213–225.

Roseman, S., F. E. Moses, J. Ludowieg & A. Dorfman. 1954. The biosynthesis of hyaluronic acid by group A streptococccus. II. Origin of the glucuronic acid. J. Biol. Chem. **206:** 665–669.

Schiller, S. & A. Dorfman. 1958. Effects of hormones on the metabolism of acid mucopolysaccharides of connective tissue. Recent Progr. Hormone Research. **14:** 427–456.

Schiller, S., M. B. Mathews, J. A. Cifonelli & A. Dorfman. 1956. The metabolism of mucopolysaccharides in animals. III. Further studies on skin utilizing C^{14}-glucose, C^{14}-acetate and S^{35}-sodium sulfate. J. Biol. Chem. **218:** 139–145.

Slack, H. G. B. 1957. Connective tissue growth stimulated by carrageenin. 2. The metabolism of sulfated polysaccharides. Biochem. J. **65:** 459–464.

Suzuki, S. & J. L. Strominger. 1959. Enzymatic synthesis of sulfated mucopolysaccharides in the hen oviduct. Biochim. Biophys. Acta. **31:** 283.

Sylven, B. & H. Malmgren. 1952. On the alleged metachromasia of hyaluronic acid. Lab. Invest. **1:** 413–431.

Tjio, J. H. & T. T. Puck. 1958. Genetics of somatic mammalian cells. II. Delineation of chromosomal constitution in tissue culture. J. Exptl. Med. **108:** 259–268.

Udupa, K. N., J. F. Woessner, Jr. & J. E. Dunphy. 1956. The effect of methionine on the production of mucopolysaccharides and collagen in healing wounds of protein-depleted animals. Surg. Gynecol. Obstet. **102:** 639–645.

Vaubel, E. 1933. The form and function of synovial cells in tissue cultures. I. Morphology of the cells under varying conditions. II. The production of mucin. J. Exptl. Med. **58:** 63–95.

Walton, K. W. & C. R. Ricketts. 1954. Investigation of the histochemical basis of metachromasia. Brit. J. Exptl. Pathol. **35:** 227–234.

Weissman, B., K. Meyer, P. Sampson & A. Linker. 1954. Isolation of oligosaccharides enzymatically produced from hyaluronic acid. J. Biol. Chem. **208:** 417–429.

Willmer, E. N. 1958. Tissue culture: the Growth and Differentiation of Normal Tissues in Artificial Media. 3rd ed. Methuen. London, England.

Windrum, G. M. 1958. The histochemical demonstration of hyaluronic acid. A.M.A. Arch. Pathol. **65:** 513–518.

Yielding, K. L., G. M. Tomkins & J. J. Bunim. 1957. Synthesis of hyaluronic acid by human synovial tissue slices. Science. **125:** 1300.

Zamenhof, S. 1957. Preparation and assay of DNA from animal tissue. *In* Methods in Enzymology. **III:** 696–704. Academic Press. New York, N. Y.

THE EFFECT OF DUSTS ON L CELLS AND PERITONEAL CELLS IN DIFFUSION CHAMBERS

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The "solubility" theory of silicosis supposes that particulate quartz, flint, and other forms of free silica are fibrogenic and produce silicosis because they slowly release a poisonous substance, that is, silicic acid, from their surfaces (King *et al.*, 1956). Although, industrially, those dusts with a reputation for not being dangerous tend to have very low silica solubilities and those that are known to be silicosis-producing have high silica solubilities, experimentally, dusts of almost identical solubilities have widely different fibrogenic powers (King *et al.*, 1956). For this and other reasons the solubility theory has been questioned increasingly.

The diffusion chamber (Prehn *et al.*, 1954) offered a new approach to the problem. Its use first showed that no fibrogenic substance was liberated from a chamber of this type containing silica (tridymite), although the dust within it dissolved in the tissue fluids at a rate comparable to that of dust lying free in the tissues (Curran and Rowsell, 1958). In further experiments it was decided to introduce cells into the chamber along with the dusts. Cells of two types were chosen: one, Earle's L strain of mouse "fibroblasts," to show whether silica would stimulate them to increased collagen formation, and the other, homologous cells harvested from the peritoneal cavity of the rat, to show whether these cells and dust combined would liberate a diffusible fibrogenic toxin or whether silica could stimulate phagocytes to fibroblastic activity within the diffusion chambers in the same way that it does when free in the tissues (Curran, 1953).

Materials and Methods

The method of constructing the diffusion chambers is described in detail elsewhere (Curran and Rowsell, 1958; Rowsell *et al.* 1960). For the experiments described in this paper, only the membrane type VC* was used, and Durofix† as the adhesive for all parts of the construction. Millipore membrane type HA (0.45 μ pore diameter) is also suitable.

In one experiment the cells implanted were Earle's mouse L cells;‡ in another they were cells harvested from the peritoneal cavities of albino rats 4 days after the injection of 2 ml. glycogen solution (0.01 mg./ml.). About 60 per cent of the peritoneal cells were of the mononuclear type and about 40 per cent were polymorphs. The numbers of cells placed in each chamber ranged from 1 to 10 million. In each experiment, the dust§ used was either silica (tridymite) or diamond, of projected area diameters of 1.8 to 5.1 μ and 0.6 to 3.5 μ, respectively; the quantity of dust in each chamber weighed from 1 to

* Millipore Corporation, Watertown, Mass.
† Rawlplug Co., Ltd., London, England.
‡ Supplied by John Paul of Glasgow University, Glasgow, Scotland.
§ Supplied by G. Nagelschmidt, Sheffield, England.

10 mg. As controls, equal numbers of empty chambers and chambers containing cells alone were used. In each of the 2 experiments the total number of chambers was 24. The chambers were placed intraperitoneally, one to each animal; mice were used for the L cells and rats for the peritoneal cells. The chambers were removed a few days to 12 weeks after insertion, fixed in 10 per cent formalin, and processed through alcohol and chloroform to paraffin. Multiple sections were cut and stained routinely with hematoxylin and eosin and for reticulin (Laidlaw's stain), collagen (van Gieson's solution and Masson's trichrome), and mucopolysaccharide (periodic acid–Schiff and Alcian blue).

Results

L cells. The chamber usually lies quite free in the peritoneal cavity of the mouse. It is enclosed, however, in a very thin fibrous membrane that forms within 7 or 8 days of implantation (Curran and Rowsell, 1958). Omentum sometimes adheres lightly to any rough area at the edge of the chamber; when inflamed omentum adheres to the central porous area, there has been a failure in the sterilizing technique, and microorganisms then are found growing inside the chamber. Sterile chambers, whether they are empty or contain dust alone, cells alone, or cells plus dust, do not excite an inflammatory reaction. Rarely, however, a chamber, although sterile, is found adherent to the inferior aspect of the liver, probably because of the relatively small size of the mouse's peritoneal cavity. In such case the growth of cells within the chamber is not affected.

Histologically, chambers empty at the time of implantation contain only watery tissue fluid. In those containing L cells, the cells multiply steadily and line the chamber within a few days. They soon form a layer several cells thick and many appear to be viable after several months (FIGURE 1). Cells die, but they fail to autolyse. Rarely, small masses of densely eosinophilic material form in close association with the cells (FIGURE 2). This substance is not argyrophil, nor does it react like collagen. Mucopolysaccharide cannot be detected within it. In the dust-containing chambers the L cells are not influenced by silica or by diamond (FIGURE 3). In particular, silica does not seem to exert either a toxic or a stimulating effect on the cells, nor does it enable them to form stainable reticulin or collagen.

Peritoneal cells. The chamber almost invariably lies free in the rat's peritoneal cavity. Its adhesion to liver is very rare. Histologically, chambers that do not receive an inoculum of cells contain only watery tissue fluid. In chambers with cells harvested from rat peritoneum most of the implanted cells obviously are dead within a few days, whether a dust is present or not; that is, nuclear staining disappears (FIGURE 4). Further autolysis often fails to occur, and the cell cytoplasm persists indefinitely as an eosinophilic mass (FIGURE 4). Occasionally a few cells retain some nuclear staining for many weeks. In a few days flattened fibroblasts appear (FIGURE 4) and soon line the inside of the chamber (FIGURE 5). Most of the tissue formed is of a loose-textured areolar type. In these chambers inoculated with both cells and dust, small quantities of the dust are found in the middle of the areolar tissue and the

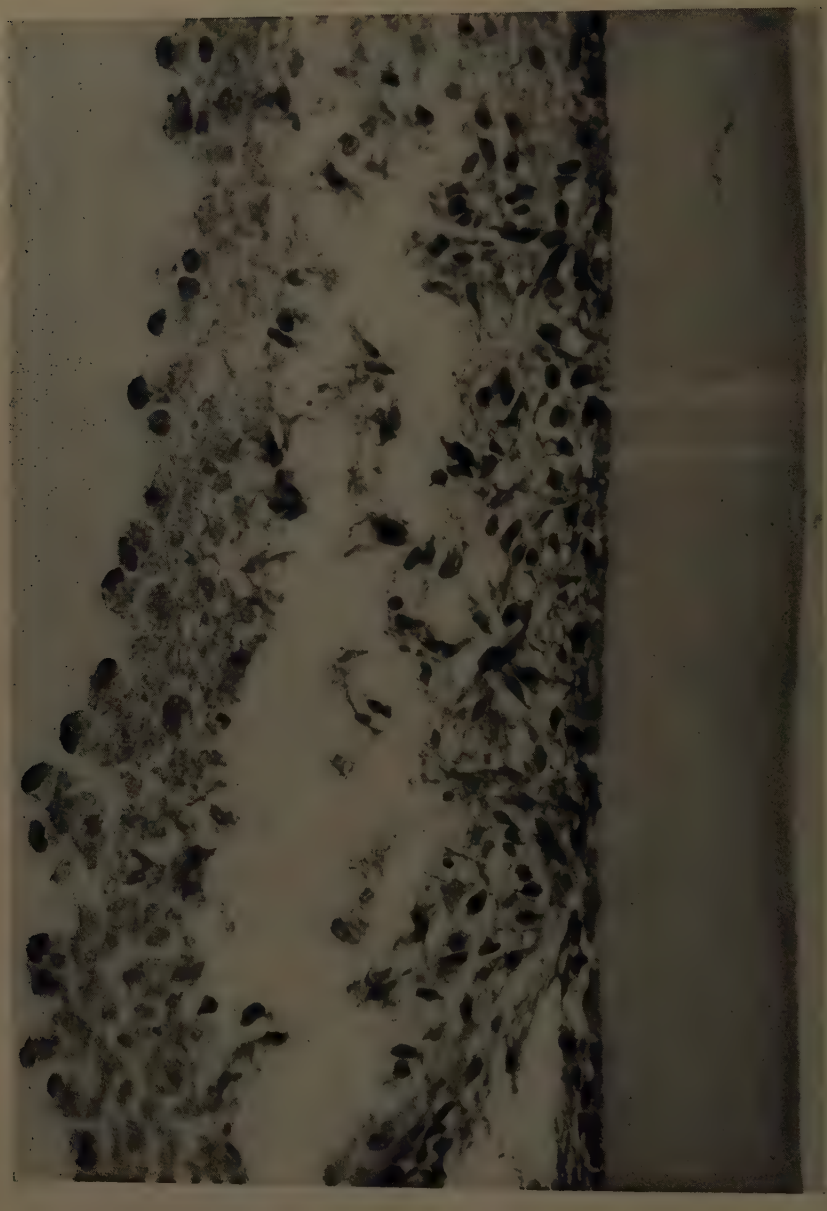

FIGURE 1. After 8 weeks, a thick layer of L cells lines the chamber. Some cells have died, but have failed to autolyse. Hematoxylin and eosin; ×180.

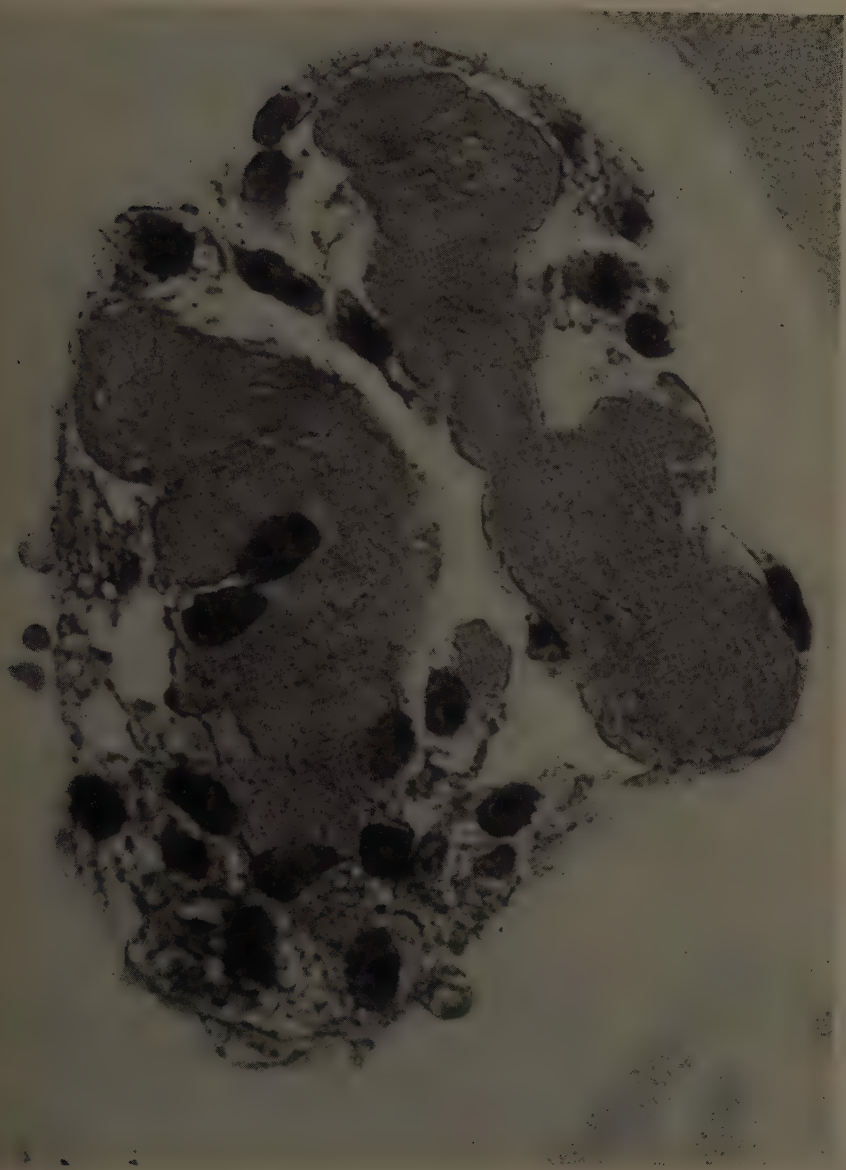

FIGURE 2. L cells after 12 weeks' growth. There is a densely eosinophilic, apparently fibrillary material in close association with the cells. It does not stain as does reticulin or collagen. Hematoxylin and eosin; ×600.

FIGURE 3. After 6 weeks' growth, a layer of L cells encloses much diamond dust, which here appears black. The dust has not influenced the growth of the cells, and there is no formation of collagen or reticulin. Hematoxylin and eosin; ×170.

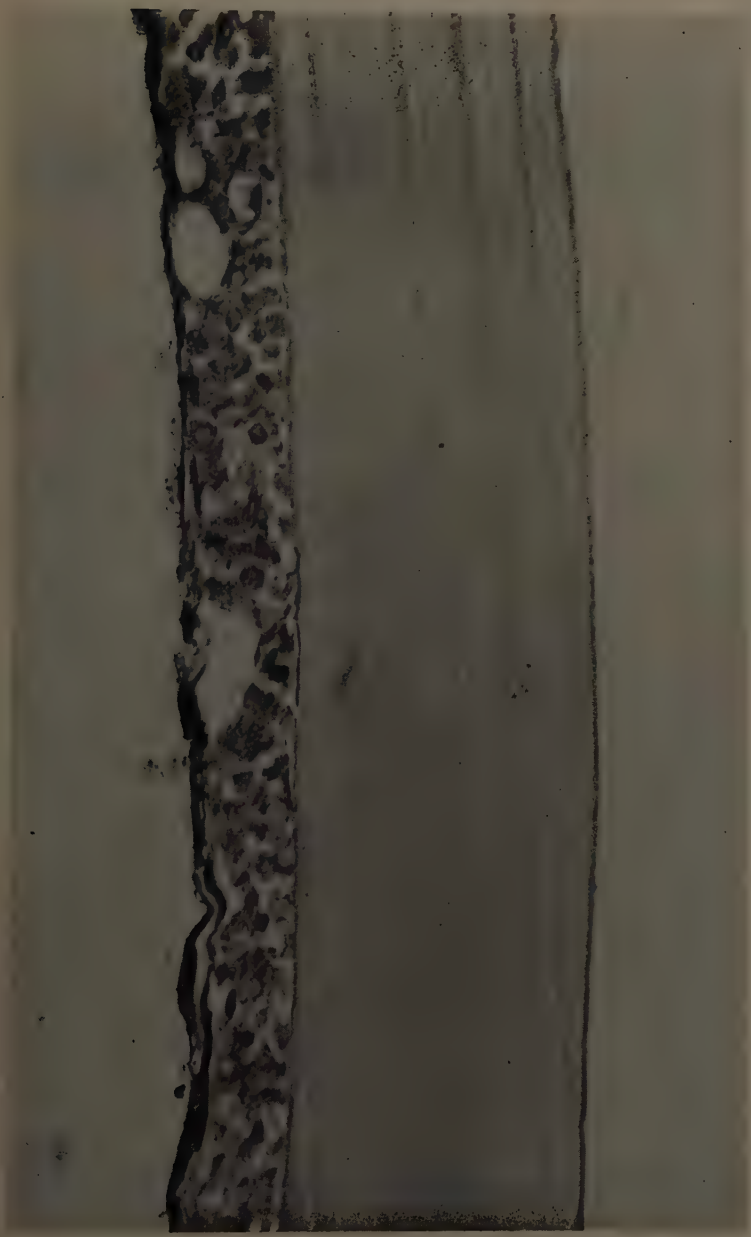

FIGURE 4. Chamber containing peritoneal cells 1 week after implantation. Fibroblasts have appeared and now line the inside of the chamber. Between them and the membrane are many dead cells, macrophages, and polymorphs of the original implant. Hematoxylin and eosin; ×300.

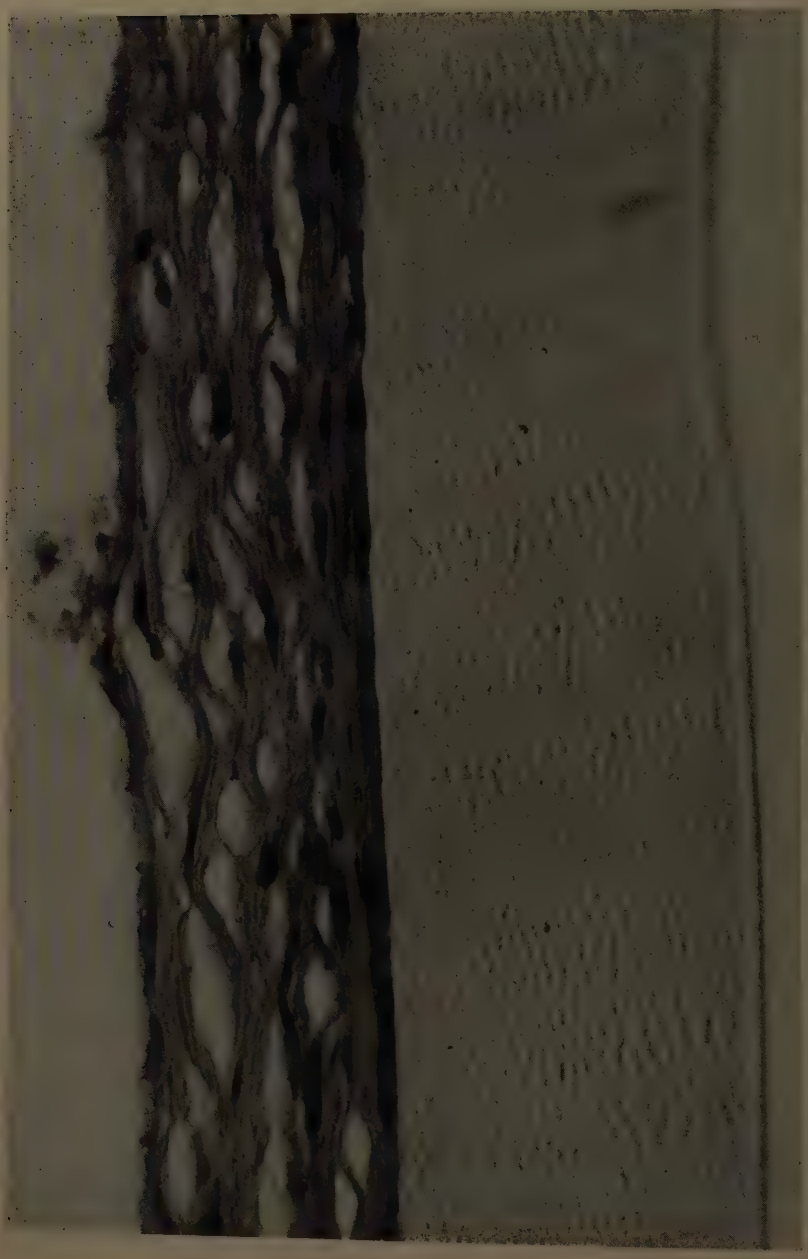

FIGURE 5. Chamber inoculated with peritoneal cells, 4 weeks after implantation. The fibroblasts have formed abundant collagen. Hematoxylin and eosin; ×600.

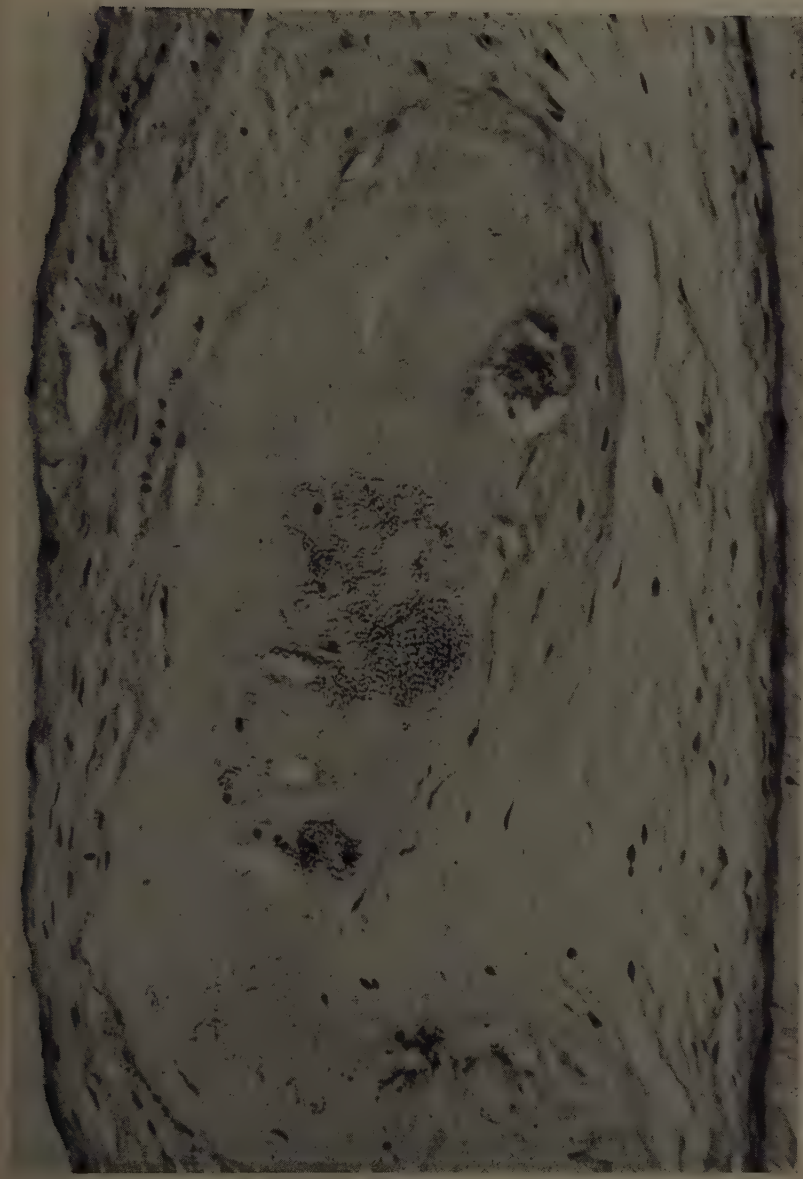

FIGURE 6. Chamber inoculated with peritoneal cells and silica 8 weeks after implantation. Refractile dust is trapped within the fibrous tissue lining the chamber. The tissue around the dust is densely hyaline and acellular. Diamond dust evokes the same response in fibroblasts. Hematoxylin and eosin; ×240.

FIGURE 7. Chamber inoculated with peritoneal cells and silica, 8 weeks after implantation. A large ovoid mass of dense fibrous tissue has formed within the chamber. Enclosed within this is dark-colored silica. Hematoxylin and eosin; ×30.

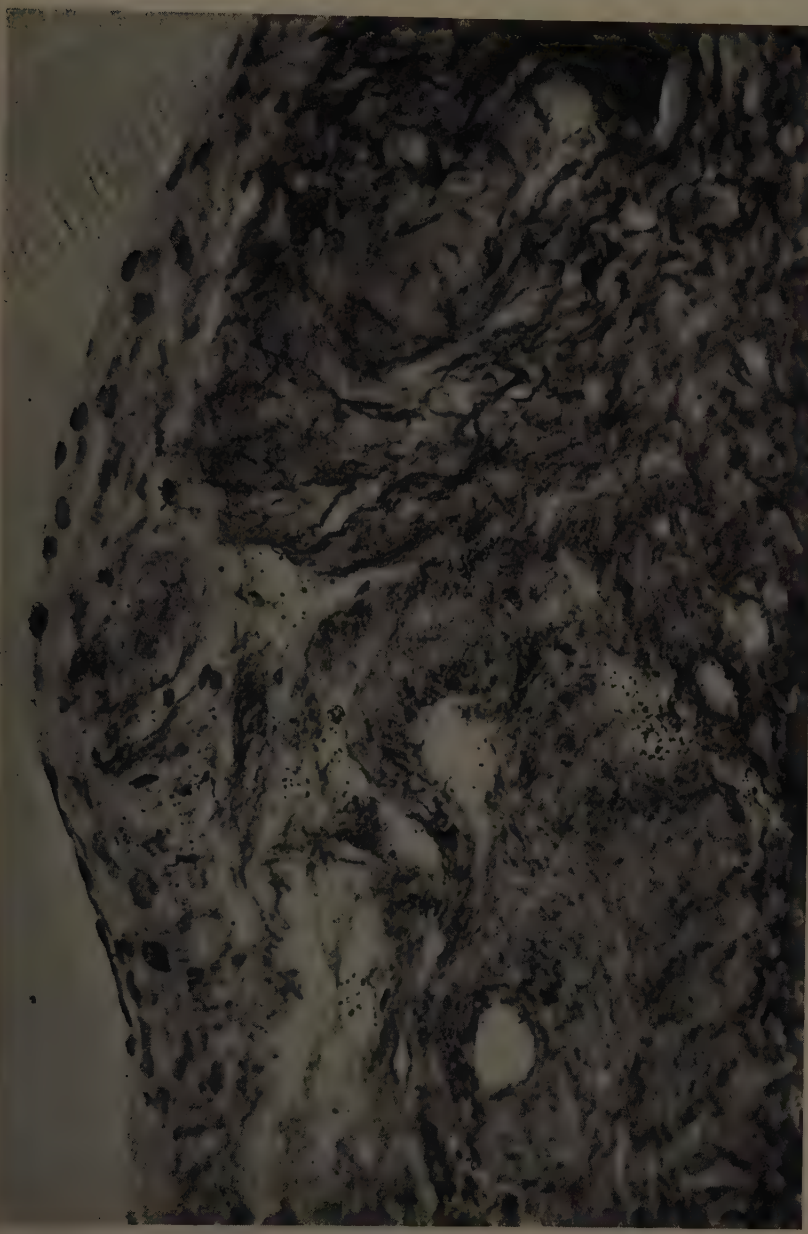

FIGURE 8. Periphery of ovoid mass illustrated in FIGURE 7. In this zone the fibers are strongly argyrophil. Fibroblast nuclei are confined to the surface. Reticulin; ×300.

fibrous tissue around the particles is dense and hyaline (FIGURE 6). The responses to both silica and diamond dust are the same: the fibroblasts apparently retreat and leave behind dense and almost acellular connective tissue. Thus they behave like osteoblasts laying down bone matrix.

Simultaneously, rounded or ovoid masses (FIGURE 7) appear, which consist to a large extent of dense, poorly cellular collagen. These structures appear within about two weeks, project into the lumen, and grow progressively until they touch the other side of the chamber. Finally they may attain diameters of several millimeters. Equally common in the silica- and in the diamond-containing chambers, relatively few occur in those chambers inoculated with cells alone. Within these nodules, few nuclei exist centrally, but around the periphery flattened fibroblasts are numerous; in this zone, too, the fibers, presumably the most recent, are more strongly argyrophil than those nearer the center (FIGURE 8). Acid mucopolysaccharide is found only in this cellular peripheral zone. It seems that the growth of the focus is peripheral and expansile.

The many sections cut from each chamber show that there is more fibrous tissue in the dust-containing than in the dust-free chambers and that there is, if anything, more in the diamond-containing chambers than in those containing silica. Dense hyaline fibrous tissue and the large ovoid or spherical fibrous masses are found almost entirely in the dust-containing chambers. After a few weeks, "free" dust, whether silica or diamond, is rarely found within any chamber, but is located within the spherical or the ovoid masses of collagen (FIGURES 7 and 8) or between the layer of fibrous tissue and the wall of the chamber.

Discussion

L cells. The inability of the L cells to form collagen greatly limited their value in these experiments. However, it was clear that silica was not toxic to these cells and that neither silica nor diamond stimulated them to proliferate or to form further proteinaceous material. If a fibroblast is defined as a cell capable of forming fibers stainable by the common histological techniques for "reticulin" or "collagen," the L cells have apparently lost the right to be called fibroblasts. It may be that during their years of growth *in vitro* they have lost some essential enzyme system. The facts that they failed to secrete stainable mucopolysaccharide and that the eosinophilic material they formed had a fibrillary structure (FIGURE 2) suggest perhaps that the defect is an inability rather to form mucopolysaccharide than to form collagen. Chemical analysis readily should answer this question.

Peritoneal cells. In the chambers inoculated with homologous peritoneal cells, proliferation of macrophages and polymorphs did not occur; the persistence of the remains of these cells within the chambers contrasted with the rapid disposal that might be expected in normal circumstances, and presumably was due to the fact that the interior of the chambers is a closed environment, effectively isolated from the reticuloendothelial system. Fibroblasts grew, however, and, since none of the peritoneal cells had the conventional elongated shape of fibroblasts at the time of implantation, it must be assumed

that peritoneal exudate contains many cells that readily become fibroblastic. In one respect this is confirmed by the rapid encapsulation of each chamber in a thin fibrous membrane; it happens to chambers lying quite free in the peritoneal cavity and having no point of adhesion to omentum or viscera.

These experiments confirmed the earlier observation (Curran and Rowsell, 1958) that silica enclosed in a diffusion chamber does not release a diffusible toxin or chemotactic substance; moreover, they showed that a silica-fibroblast mixture does not do so either. Since there was no detectable proliferation of phagocytes, the possibility that a phagocyte-silica mixture liberates a fibrogenic substance (Fallon, 1937) was not tested.

It is difficult to understand how a chemically inert substance such as diamond can lead to the production of so much hyaline collagen. It should be noted that this ability to react to "inert" dusts is not peculiar to fibroblasts of peritoneal origin, for it is found in fibroblasts grown from fetal and adult rat liver (Curran and Rowsell, unpublished observations). In this context the observation that sheets of such relatively inert materials as mica and plastic stimulate fibroblasts to such proliferation that sarcoma often ensues (Oppenheimer *et al.*, 1958) may be relevant. It seems that fibroblasts in contact with any surface, regardless of its chemical nature, are always disturbed and, of course, the finely particulate materials used in the present experiments remain extracellular and thus present large surface areas to the fibroblasts. It is possible that reputedly nonfibrogenic dusts such as diamond stimulate the formation of fibrous tissue in certain pathological states in man. Certainly, in rats a mixture of quartz and inert dust produces much more fibrous tissue than does quartz alone (Ray *et al.* 1951); it may be that the phagocytes are killed by the quartz and that the fibroblasts that penetrate the quartz "barrier" then are stimulated by the "inert" dust.

The lack of a toxic effect of silica on peritoneal fibroblasts and L cells is interesting in view of this dust's profoundly irritant effect on normal tissues. It contrasts with the extraordinary susceptibility of polymorphs and macrophages to the same dust lying free in the tissues. Perhaps the silica dust must be ingested before a toxic effect may be produced.

The large spherical or ovoid masses of fibrous tissue are largely dust-induced, but their formation is somewhat obscure. The most obvious explanation is that the particles infiltrate between the sheet of fibroblasts and the wall of the chamber and thereby lessen the cells' adhesiveness to the membrane. More probably, the dense fibrous tissue formed in response to the dust contracts greatly as it matures, and pulls the layer of tissue off the membrane; the tissue then would tend to roll up into a rounded mass that thereafter may grow steadily in an expansile and peripheral manner.

Although these chambers appear to be useless in assessing the fibrogenic qualities that a dust may be expected to display in a normal environment, they probably are of considerable value in the study of fibrogenesis, for the reason that all the tissue is formed *de novo*.

References

Curran, R. C. 1953. J. Pathol. Bacteriol. **64:** 271.
Curran, R. C. & E. V. Rowsell. 1958. *Ibid.* **76:** 561.

FALLON, J. T. 1937. Can. Med. Assoc. J. **36:** 223.
KING, E. J., S. H. ZAIDI & G. NAGELSCHMIDT. 1956. Arch. Ind. Health. **13:** 133.
OPPENHEIMER, B. S., E. T. OPPENHEIMER, A. P. STOUT, M. WILLHITE & I. DANISHEFSKY. 1958. Cancer. **11:** 204.
PREHN, R. T., J. M. WEAVER & G. H. ALGIRE. 1954. J. Natl. Cancer Inst. **15:** 509.
RAY, S. C., E. J. KING & C. V. HARRISON. 1951. Brit. J. Ind. Med. **8:** 68.
ROWSELL, E. V., G. NAGELSCHMIDT & R. C. CURRAN. J. Pathol. Bacteriol. In press.

THE EFFECTS OF PAPAIN ON CARTILAGE *IN VIVO*: FACTORS INFLUENCING THE DISTRIBUTION OF PAPAIN PROTEASE FOLLOWING INTRAVENOUS INJECTION*

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The Effects of Papain on Cartilage in Vivo

It has been shown that widespread depletion of cartilage matrix can be produced in young rabbits by the intravenous injection of the water-soluble components of crude papaya latex.[1] Within a few hours after the injection, the effect on cartilage is evinced by drooping of the rabbits' ears, which results from a loss of the normal rigidity of the supporting cartilaginous plates. In this and in all other cartilage examined, including laryngeal, tracheal, costal, articular, and epiphyseal, histological changes appear within a few hours and reach a maximum within 24 hours. The normal basophilic and metachromatic staining properties of the matrix are lost, and the intercellular substance is reduced in amount.[1,2]

In the cartilage plates of the ears, normal rigidity is recovered within a few days, but recovery of the normal histological appearance of cartilage throughout the body may take 3 weeks. Reconstitution of the matrix is largely prevented by the administration of cortisone, hydrocortisone, or prednisolone, apparently because of a direct local action of the steroids on cartilage.[1,3]

It has been shown that this effect of crude papain on cartilage is associated with the release of large amounts of chondroitin sulfate into the circulation.[4,5] In all probability the changes are produced by a direct enzymatic breakdown of chondromucoprotein in the matrix.

The purified crystalline protease from crude papain has been found to produce similar gross and histological changes in cartilage *in vivo* when injected intravenously.[6] However, this effect is seen only when the enzyme is inactivated by oxidation (dialysis) prior to injection. If injected in the fully active, reduced form, crystalline papain has little or no effect on cartilage *in vivo*.[6] On the other hand, isolated plates of cartilage are depleted rapidly when exposed *in vitro* to the fully active enzyme.

To explain this anomaly, it was postulated that the failure of active crystalline papain to produce depletion of cartilage *in vivo* resulted from a prompt interaction, following injection, with material in the circulation that prevented entry of the enzyme into cartilage; inactive papain, on the other hand, remained free to diffuse into cartilage, where it was activated and produced depletion of the matrix.

This hypothesis was strengthened by the observation that ear cartilage removed from rabbits 15 min. after an intravenous injection of inactive crystalline papain and incubated at 37° C. in phosphate buffer showed progressive

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depletion of the matrix; this effect was prevented by addition of thiol antagonists to the buffer solution. In cartilage removed 15 min. after an injection of active crystalline papain the matrix remained intact throughout subsequent incubation in buffer alone or in buffer containing cysteine.[6] These observations are strong evidence that papain is taken up by cartilage and is responsible for the changes observed there.

In the present report, evidence is presented showing that depletion of cartilage matrix *in vivo* produced by the injection of inactive papain protease is accompanied by the release of chondoitin sulfate into the circulation and that no such release occurs after injection of the fully active enzyme. Additional evidence is offered in support of the hypothesis that the failure of active papain to produce *in vivo* changes in cartilage results from interaction with material in the circulation, and an account is given of preliminary observations on the distribution of papain *in vivo* and factors in serum that influence this distribution.

Turbidimetric Determination of Chondroitin Sulfate in Rabbit Serum Following Administration of Crystalline Papain

In the following experiments, the concentration of chondroitin sulfate in serum was measured by a turbidimetric procedure using the trivalent cation hexammine cobaltic chloride, which in media of low ionic strength combines with anionic materials such as chondroitin sulfate to form insoluble complexes. It has been shown that the resulting increase in turbidity is proportional to the concentration of chondroitin sulfate added *in vitro*. The details of the method, together with data on its specificity and limitations, have been reported elsewhere.[7]

Albino rabbits weighing 800 to 1000 gm. were given intravenous injections of either active or inactive crystalline papain, or given no injection, and were bled by cardiac puncture immediately and again at intervals in the following 24 hours to provide serum for determination of chondroitin sulfate.

The crystalline papain* was supplied as a solution containing 20 mg./ml. in phosphate buffer with 0.03 *M* cysteine. After dilution, this was dialyzed for 24 hours at 4° C. with a two hundredfold volume of phosphate buffer. This resulted in removal of excess cysteine and in almost complete inactivation of the enzyme, presumably by oxidation to the disulfide form, as measured by hydrolysis of benzoyl-1-arganinamide according to the method of Davis and Smith.[8]

Aliquots of this material were then either (1) mixed with an equal volume of 0.01 *M* cysteine HCl in phosphate buffer and incubated at 37° C. for 15 min. prior to injection (active reduced papain) or (2) mixed with an equal volume of buffer alone and incubated at 37° C. for 15 min. prior to injection (inactive oxidized papain).

All injections consisted of 5 mg. of the protease in a volume of 1 to 2 ml., and were given by ear vein.

Data from these experiments are collected in TABLE 1. It was found that a marked increase in serum concentration of cobalt-precipitable material occurred during the 24-hour period after an injection of inactive crystalline pa-

* Obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio.

pain. Little or no increase occurred in the amount of cobalt-precipitable material in the serum of uninjected rabbits or of rabbits given active crystalline papain. This constitutes further evidence that fully active crystalline papain has little or no effect upon cartilage *in vivo* following intravenous injection.

Depletion of Cartilage Matrix by Papain in Vitro

Isolated plates of rabbit ear cartilage undergo rapid depletion of the matrix on incubation at 37° C. in solutions containing crystalline papain.[6] In the

TABLE 1
EFFECT OF ACTIVE AND INACTIVE CRYSTALLINE PAPAIN *IN VIVO*

Preparation injected	No. rabbits	Drooping of ears at 24 hours	Chond.sulf. equivalent* in serum at 24 hours (mg./ml.)
Inactive crystalline papain, 5 mg.	40	+ to ++++	2.0 to 6.0
Active crystalline papain, 5 mg.	16	− to +	0.0 to 1.3
Nil	21	− to ±	0.0 to 0.1

* Concentration of bovine nasal chondroitin sulfate in normal rabbit serum giving same turbidity with hexammine cobaltic chloride.

TABLE 2
EFFECT OF ACTIVE CRYSTALLINE PAPAIN ON CARTILAGE *IN VITRO*

Concentration of papain in medium* (μg./ml.)	Changes in cartilage after 4 hours at 37° C.		
	Gross softening	Histological depletion	Chond. sulf. equivalent† in medium (μg./ml.)
10	+++	++	60
8	++	++	
6	+	+	
4	+	±	25
2	+	±	
1	±	±	
0.5	−	−	1
0.2	−	−	
Nil	−	−	1

* Volume of medium = 40 ml., containing cysteine 0.005 *M* and Versene 0.001 *M*.
† Concentration of bovine nasal chondroitin sulfate giving same turbidity with hexammine cobaltic chloride.

following experiments this effect of papain was studied further by using a range of concentrations of the enzyme to determine the minimal effective concentration.

The skin and connective tissues were stripped from ear cartilage plates freshly removed from 800-gm. rabbits. The plates were immersed for 4 hours in 40 ml. phosphate buffer containing 0.005 *M* cysteine and crystalline papain in concentrations ranging from 0.2 to 10.0 μg./ml. The plates then were examined for loss of rigidity, and sections were obtained for histological study. Aliquots of the media were analyzed for cobalt-precipitable material.

It was found that as little as 2 μg./ml. of crystalline papain produced a de-

tectable loss of rigidity; unequivocal histological changes were present in cartilage incubated in concentrations of papain of 6 μg./ml. or greater (TABLE 2). According to measurements of increased turbidity produced by addition of hexammine cobaltic chloride to dialyzed diluted aliquots of the media, the

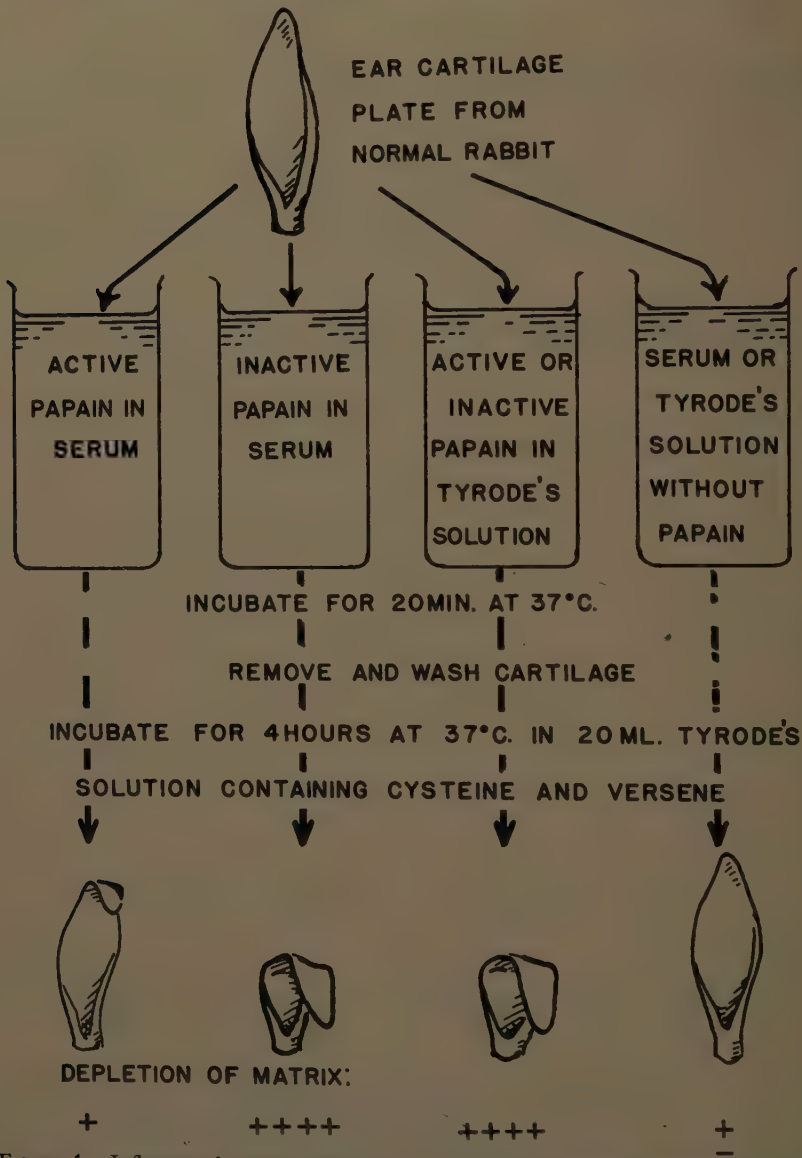

FIGURE 1. Influence of normal rabbit serum on entry of papain protease into cartilage *in vitro*. Concentration of papain = 100 μg./ml.; concentration of cysteine = 0.005 *M*; concentration of Versene = 0.001 *M*. In each comparison of the effects of active and inactive papain, the two plates of cartilage used were obtained from one rabbit.

material so precipitated would represent approximately 2.4 mg. of chondroitin sulfate released from the cartilage incubated in medium containing 10 μg. papain per milliliter. This estimate is based, of course, on the assumption that all the precipitable material is chondroitin sulfate; however, some protein may be included.

For a reproduction *in vitro* of conditions *in vivo*, the procedure shown schematically in FIGURE 1 was devised. Isolated plates of cartilage, prepared as described above, were immersed for 20 min. at 37° C. in 1 of 4 solutions (FIGURE 1). The papain had been mixed with the prewarmed serum or Tyrode's solution just before the plates of cartilage were inserted. After incubation in these mixtures, the plates were washed and transferred to tubes containing 20 ml. Tyrode's solution with 0.005 *M* cysteine and 0.001 *M* Versene, and incubated for 4 hours at 37° C.

TABLE 3

EFFECT OF NORMAL RABBIT SERUM ON ENTRY OF ACTIVE AND INACTIVE PAPAIN INTO CARTILAGE *IN VITRO*

Medium used in initial 20-min. incubation at 37° C.	Changes after washing and incubating for 4 hours at 37° C. in 20 ml. Tyrode's solution with 0.005 *M* cysteine and 0.001 *M* Versene		
	Loss of rigidity	Depletion of matrix	Chond. sulf. equivalent in medium* (mg./ml.)
Inactive papain in Tyrode's solution	+++	++++	1.0
Active papain in Tyrode's solution	+++	++++	1.25
Inactive papain in serum	+++	++++	1.0
Active papain in serum	+	+	0.45
Tyrode's solution only	±	−	0.02
Serum only	±	−	0.01

* Concentration of bovine nasal chondroitin sulfate giving same turbidity with hexammine cobaltic chloride.

At this stage the plates of cartilage initially exposed to either active or inactive papain in Tyrode's solution showed approximately equal loss of rigidity, histological depletion of matrix, and release of chondroitin sulfate, as measured by the amount of cobalt-precipitable material in the medium (TABLE 3).

The plate initially exposed to inactive papain in serum showed similar loss of rigidity and depletion of matrix after 4 hours' incubation in Tyrode's solution with cysteine, and released into the medium as much cobalt-precipitable material as did the plates initially exposed to papain in Tyrode's solution.

In contrast, the cartilage initially exposed to fully active papain in serum subsequently showed only slight loss of rigidity and histological evidence of depletion of matrix (FIGURE 2), and released into the medium substantially less cobalt-precipitable material than did the contralateral ear exposed initially to inactive papain (TABLE 3).

In the same experiment 2 additional plates of cartilage were incubated in serum or Tyrode's solution without papain for 20 min., and were then washed and incubated in Tyrode's solution with 0.005 *M* cysteine for 4 hours at 37° C.

At the end of this period they neither showed loss of rigidity or histological changes nor released a significant amount of cobalt-precipitable material.

This experiment, with minor modifications, has been repeated twice with essentially the same result.

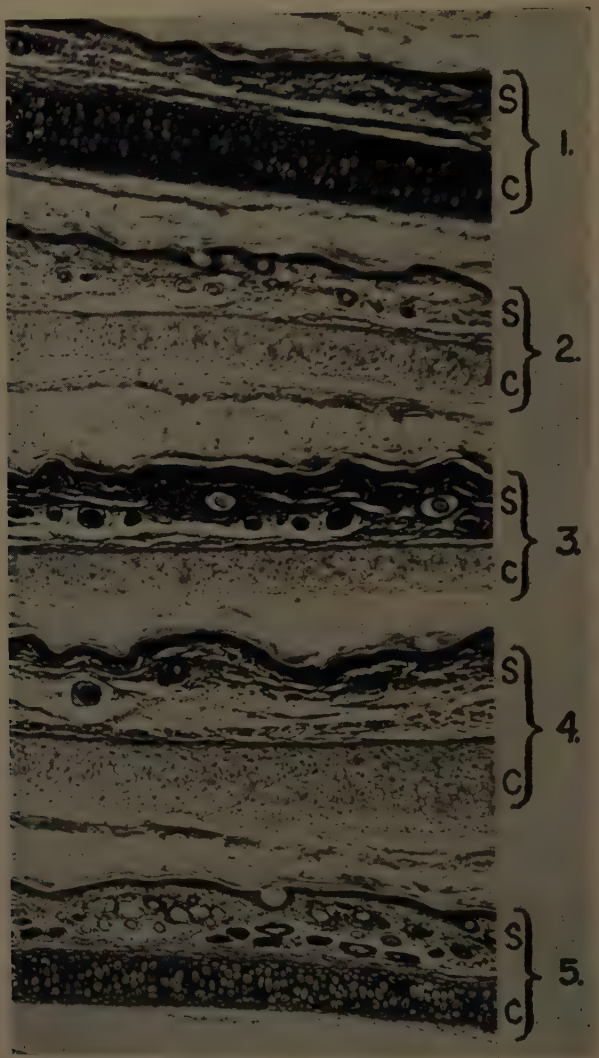

FIGURE 2. Histological appearance of cartilage plates following exposure to papain *in vitro*. For complete description of procedure see text and FIGURE 1. The cartilage plates were mounted together in one block to ensure uniform handling during sectioning and staining. *S* = skin on inner surface of ear plate; *C* = cartilage plate. In *C1* the appearance of the matrix is normal; in *C2*, *3*, and *4* the matrix shows almost complete loss of basophilia; in *C5* the matrix is only slightly less basophilic than normal. The media in which cartilage was initially incubated are designated by the numbers: *1*, Tyrode's solution; *2*, inactive papain in Tyrode's solution; *3*, inactive papain in normal rabbit serum; *4*, active papain in Tyrode's solution; *5*, active papain in normal rabbit serum. × 25.

Rates of Disappearance from the Circulation of Active and Inactive Papain Following Intravenous Injection

In view of the foregoing it was concluded that active and inactive papain might differ in their rates of disappearance from the circulation following intravenous injection. This was studied first by using a specific precipitating antiserum to papain prepared in rabbits.

Preliminary tests showed that active and inactive papain were quantitatively similar to antigens in the quantitative precipitin reaction (FIGURE 3).

Rabbits were given injections of either active or inactive crystalline papain in doses ranging from 2 to 5 mg. intravenously, and were bled by cardiac puncture at intervals of up to 24 hours. The sera obtained were tested with papain antisera by double diffusion in agar. By this method it was found that the concentration of papain in serum a few minutes after the injection was in the range of 40 to 100 μg./ml., and as much as 10 μg./ml. 24 hours later (FIGURE 4). However, no difference was demonstrable between the rates of disappearance of active and inactive papain. Both preparations were present chiefly in the plasma fraction of blood, little or none being present in the washed cells. Immunoelectrophoresis of serum from papain-treated rabbits showed that the enzyme moved electrophoretically with the alpha$_2$ globulin fraction, whether injected in the active or the inactive form.

In view of the possibility that differences in disappearance between active and inactive papain might be too small to be detected by immunological techniques, further studies were made using papain labeled with I^{131}. A solution of 100 mg. crystalline papain was dialyzed for 24 hours with physiological saline to remove excess cysteine and to oxidize the enzymatically active thiol group of the molecule. To this was added 1.0 ml. carbonate buffer at *p*H 10.0, 0.05 *M*. A solution of carrier-free I^{131}, 2 mc. in 2.0 ml., was added to 1 ml. of KI_3 solution,* and the mixture added to the buffered solution of papain. After standing for 20 min. at 4° C., the reaction mixture was transferred to dialysis tubing and dialyzed with 4 changes of physiological saline, 1 l., at 4° C.

This preparation was stored at 4° C.; aliquots used in experiments were dialyzed for 18 to 24 hours and, after warming to 37° C., centrifuged for 15 min. at 2500 rpm to remove trace amounts of insoluble material.

In vitro tests showed that 80 per cent of the original proteolytic activity could be restored following iodination by the addition of cysteine and Versene, giving, respectively, 0.005 and 0.001 *M* concentrations. Solutions of labeled papain so treated, or following incubation at 37° C., or following treatment with iodoacetamide, contained no significant amount of dialyzable radioactivity. This showed that the I^{131} bound to the enzyme remained attached despite the usual activating and inactivating procedures. All of the radioactivity in solutions of labeled papain was precipitated by a specific antiserum to papain.

Rabbits weighing approximately 1000 gm. were given injections of labeled papain, either active or inactive, in doses of either 2 or 4 mg. The rates of disappearance were similar for both enzyme preparations at both dosage levels, when the serum radioactivity was expressed as a percentage of that found 1

* I_2 0.123 gm./100 ml., KI 0.157 gm./100 ml.

min. after injection of the labeled papain (FIGURE 5). The material precipitated from samples of these sera by rabbit antipapain serum contained approximately 85 per cent of the total radioactivity, and it was evident that any difference between the rates of disappearance of active and inactive papain

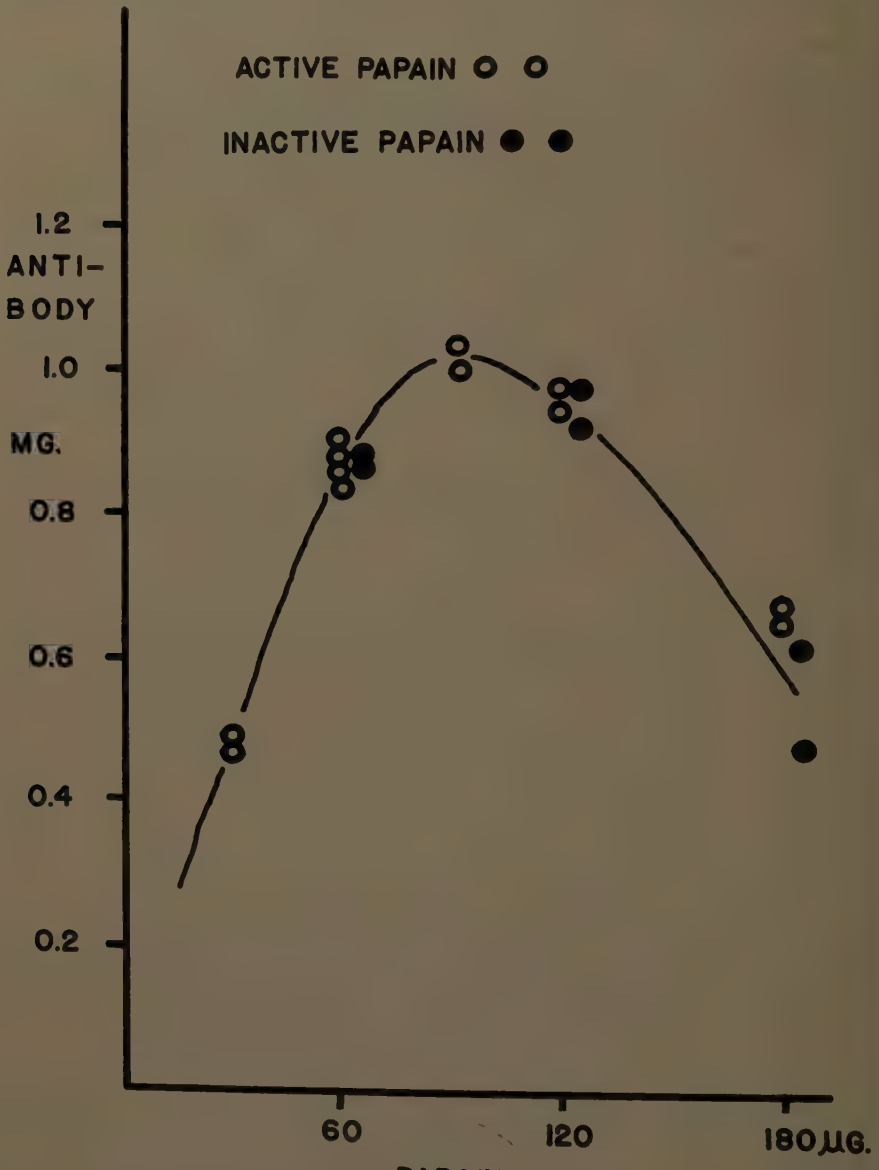

FIGURE 3. Quantitative precipitin test on rabbit antipapain serum to compare quantitative behavior of active and inactive papain as antigens. The washed precipitates were redissolved in 0.25 *M* acetic acid. The protein concentration was determined from optical density at 280 mμ.

must be very small indeed, at least after injections in the range of 2 to 4 mg./kg.

Further studies are in progress to explore the possibility that a difference will be more obvious when larger amounts of papain are injected. However,

FIGURE 4. Double diffusion in agar as a method of detecting papain in rabbit serum following intravenous administration of active and inactive preparations. The Ouchterlony plate was photographed 3 days after the 2 arrays of wells were filled as follows:

A1, and A4, dialyzed (inactive) papain in saline.
A2, reduced (active) papain with cysteine in saline.
A3, iodoacetamide-treated (once) papain in saline.
A5, iodoacetamide-treated (twice) papain in saline.
A6, iodoacetamide-treated (twice) papain with cysteine in saline.
In each case concentration of papain = 100 μg./ml.
A12 and B 12, rabbit antiserum to crystalline papain.
B7, dialyzed (inactive) papain, 100 μg./ml. in saline.
B13, dialyzed (inactive) papain, 100 μg./ml. in normal rabbit serum.
B8 and B11, serum from 1 kg. of rabbit 10 min. and 90 min. after I.V. injection of 5 mg. inactive papain.
B9 and B10, serum from 1 kg. rabbit 10 min. and 90 min. after I.V. injection of 5 mg. active papain.
The single line of identity in A shows that active and inactive papain react in a similar fashion with the same specific antibody.
In B, the lines between the center well, 12, and the peripheral wells, 8, 9, 10, and 11, demonstrate the presence of papain in the serum of 2 rabbits 10 min. and 90 min. after injection of active and inactive papain. The distance between the peripheral well and the corresponding line is a measure of the concentration of papain in that well; in this example, the fall in concentration between 10 and 90 min. following injection of active papain (B8 and 11) is the same as that following injection of inactive papain (B9 and 10).

it also seemed possible that differences exist between active and inactive papain in their distribution in serum.

In 2 rabbits, the radioactivity of ear cartilage was measured 30 min. after injections of I^{131}-labeled papain; it was estimated that the amount of papain in cartilage did not exceed 1 μg./gm. wet weight. This figure is in keeping with that estimated on the basis of immunological procedures using extracts of cartilage and specific antiserum to papain, which indicated that the concentration in this tissue was less than 10 μg./gm. of wet cartilage.

To explore possible differences in distribution between active and inactive papain in serum, samples from rabbits injected with active and inactive papain and samples of serum to which papain was added *in vitro* were fractionated by starch block electrophoresis.[9] As shown in FIGURE 6, the distributions of active and inactive papain in serum were essentially similar, whether mixed *in vitro* or *in vivo*, most of the radioactivity being present in the fractions cor-

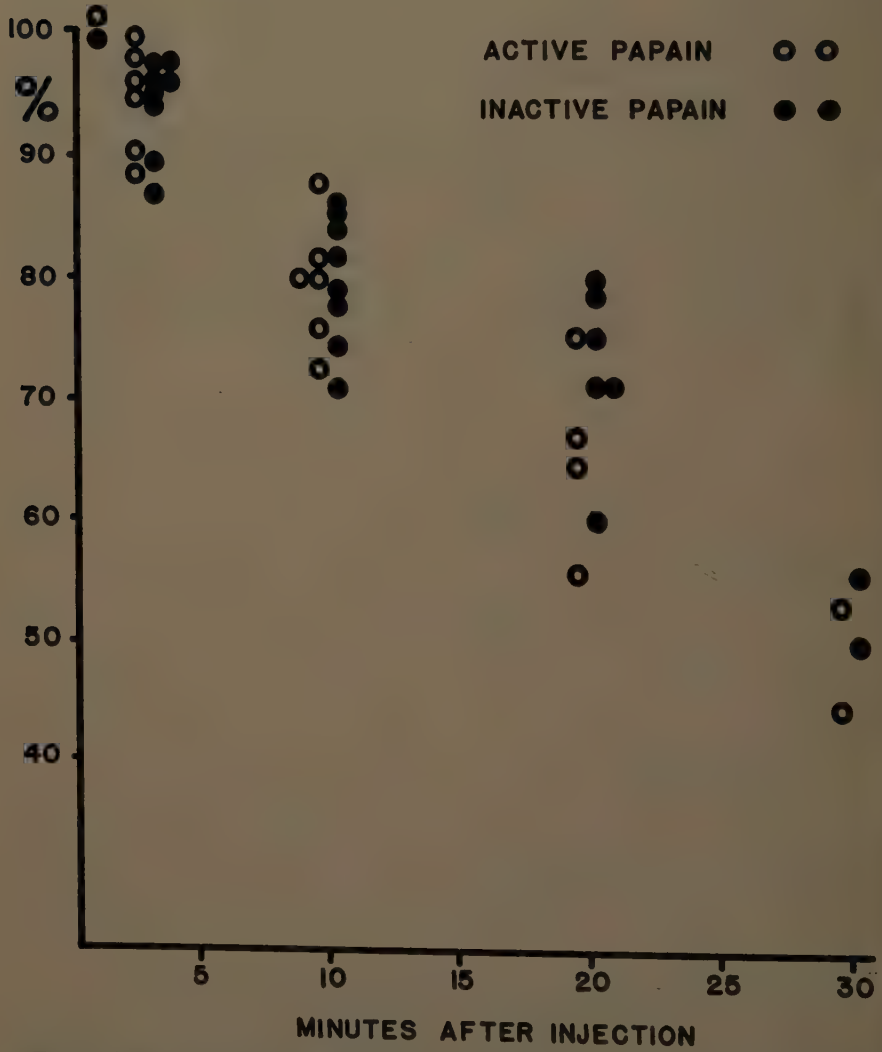

FIGURE 5. Rates of disappearance of I^{131}-labeled papain from the circulation following intravenous injection in rabbits. Concentrations at intervals after injection in individual rabbits are expressed as percentages of the radioactivity (cpm/ml.) in serum obtained one minute after completion of I.V. injection of I^{131}-labeled papain. For clarity, only 2 of the 14 initial (100 per cent) concentrations are shown.

responding to the alpha globulin zones. Papain in saline in parallel electrophoresis on starch had little or no mobility at this *p*H, and remained at the origin.

Thus it appeared that both active and inactive papain in concentrations approximating 50 μg./ml. were bound to proteins in the alpha globulin region; additional studies with more prolonged electrophoresis and the preparation

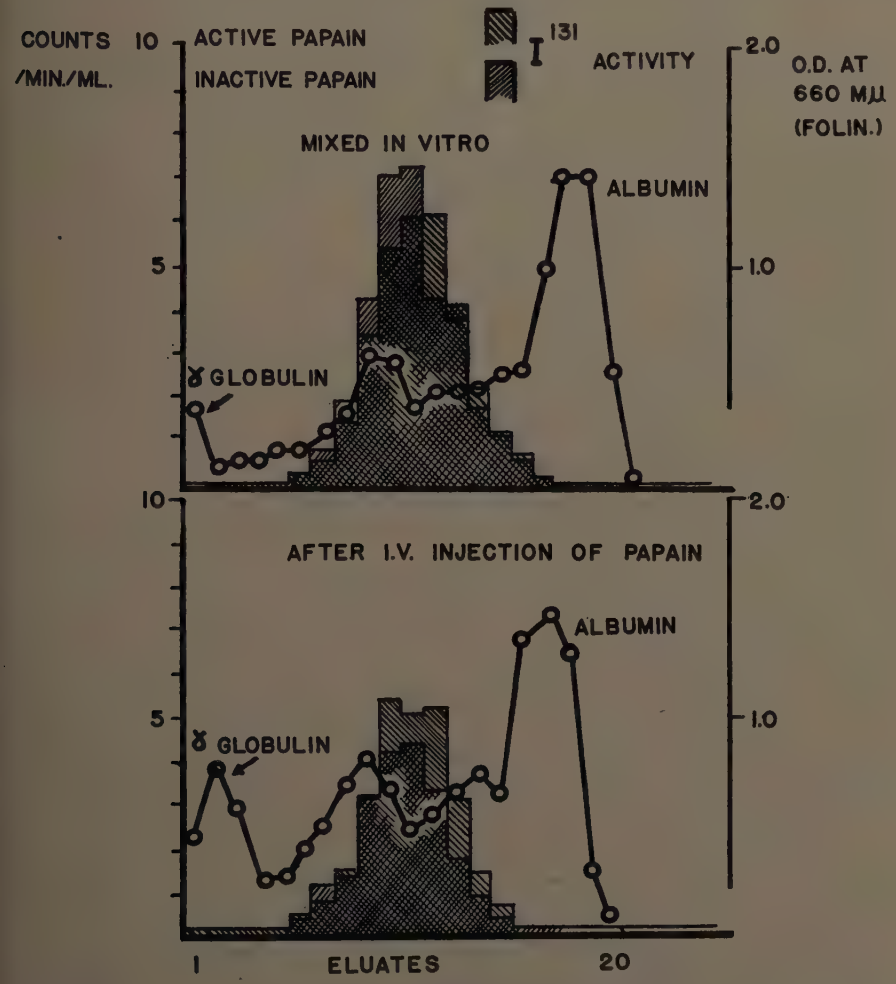

FIGURE 6. Distribution of protein and radioactivity in eluates from starch block electrophoresis of rabbit serum containing I^{131}-labeled papain. *Upper chart:* radioactivity (*left, ordinate, hatched blocks*) and protein concentration (*right ordinate, circles*) in eluates from starch block electrophoresis of rabbit serum to which active or inactive I^{131}-labeled papain was added to give a concentration of 50 μg./ml.; the mixture was incubated at 37° C. for 30 min. prior to electrophoresis at *p*H 8.6.

Lower chart: electrophoretic distribution of radioactivity and protein in serum obtained from rabbits 30 min. after I.V. injection of I^{131}-labeled papain; the concentration of papain was approximately 40 μg./ml. of serum.

of a larger number of fractions from the same block showed a peak concentration corresponding with the alpha$_2$ globulin fractions.

Electrophoresis on starch of 3 fractions of normal serum after addition of labeled papain and incubation at 37° C. for 30 min. showed complete binding with alpha$_2$ globulin, while no papain was bound by gamma globulin and only 20 per cent was bound by the albumin fraction.

Discussion

Following the intravenous injection into rabbits of inactive crystalline papain, widespread depletion of cartilage matrix is seen, accompanied by a marked increase in the amount of cobalt-precipitable material, presumed to be chondroitin sulfate, in serum.

The demonstration that no such increase occurs in the amount of cobalt-precipitable material in serum of rabbits following the intravenous injection of active crystalline papain constitutes additional evidence that in this form the enzyme fails to enter cartilage *in vivo*.

The hypothesis that this paradoxical difference is due to the prompt interaction *in vivo* between active crystalline papain and a factor or factors in the circulation that hinder its entry into cartilage has been examined with an *in vitro* model. It has been found that cartilage exposed to inactive papain in serum for a short period subsequently shows depletion of its matrix, whereas only slight depletion occurs following exposure of cartilage to active papain in serum. Since cartilage undergoes a depletion following exposure to active papain in Tyrode's solution that is equal to or greater than that occurring following exposure to inactive papain in Tyrode's solution, it may be concluded that normal rabbit serum has the property of impairing the entrance of active papain into cartilage. The simplest explanation that can be offered for this is that the process may be one of enzyme-substrate combination.

On the basis of these observations it was expected that inactive papain would disappear from the circulation more rapidly than active papain. However, neither immunological procedures nor studies with I^{131}-labeled papain revealed a consistent difference between active and inactive papain injected intravenously in doses of 2 to 4 mg./kg. In view of the fact that changes can be produced in cartilage *in vitro* using as little as 2 μg. of papain per milliliter of surrounding medium, it is entirely possible that an equivalent amount passing into cartilage from the circulation would not appreciably alter its concentration in serum. On the basis of preliminary observations using I^{131}-labeled papain it appears that, even when a sufficient amount of inactive papain enters cartilage and produces depletion, the concentration in that tissue is only a fraction of that in serum. In other words, papain is not selectively concentrated in cartilage. It is not known why, in spite of this, cartilage is the only tissue in which histological changes are regularly demonstrable following an intravenous injection of papain. However, it may well be that in cartilage alone the breaking of relatively few chemical bonds leads to pronounced structural changes.

Electrophoretic studies of the distribution of I^{131}-labeled papain in serum following *in vitro* or *in vivo* mixing have shown that both active and inactive

papain are selectively bound in the region of the alpha$_2$ globulin. The relationship between this selective binding of papain and the differences in behavior of intravenously administered active and inactive papain is not known, but our data do not preclude the possibility that active papain is bound more rapidly by this fraction of serum and that with inactive papain a small proportion passes into cartilage in the first few minutes following injection. Alternatively, both forms of the enzyme may be bound with equal efficiency by this fraction of serum, and the changes in cartilage would be the result of the passage from the circulation of a portion of inactive papain in excess of the amount that may be bound by this fraction, while a similar excess in the case of active papain may combine in the circulation through its active site with substrates such as albumin.

The existence in normal serum of a fraction to which papain is selectively bound may exemplify a mechanism whereby the tissues are protected against injury from potentially harmful enzymes that have entered the circulation.

Summary

Depletion of cartilage matrix *in vivo* following injection of inactive crystalline papain is associated with increased amounts of material in serum that can be precipitated by hexammine cobaltic chloride. The intravenous injection of active crystalline papain, which does not produce *in vivo* depletion of cartilage matrix, is not followed by any such increase in the level of chondroitin sulfate in serum.

The depletion of cartilage matrix *in vivo* by papain is due to a small proportion of the amount injected intravenously; papain is not concentrated in cartilage *in vivo*.

Normal rabbit serum impairs the entry of active papain into cartilage *in vitro*, but does not have a comparable effect on the entry of inactive papain into cartilage.

After a single intravenous injection of 2 to 4 mg. of papain no difference can be detected in the rates of disappearance from the circulation of papain injected in the active or inactive form.

In serum, papain is bound to protein in the region of the alpha$_2$ globulin. This electrophoretic localization was similar for active and inactive papain and was demonstrated following intravenous injection and *in vitro* mixing.

References

1. Thomas, L. 1956. Reversible collapse of rabbit ears after intravenous papain, and prevention of recovery by cortisone. J. Exptl. Med. **104:** 245.
2. Spicer, S. S. & J. H. Bryant. 1957. Cartilage changes in papain treated rabbits. Am. J. Pathol. **33:** 1237.
3. McCluskey, R. T. & L. Thomas. 1959. The removal of cartilage matrix *in vivo* by papain. Prevention of recovery with cortisone, hydrocortisone and prednisolone by a direction action on cartilage. Am. J. Pathol. **35:** 819.
4. Bryant, J. H., I. G. Leder & D. Stetten, Jr. 1958. The release of chondroitin sulfate from rabbit cartilage following the intravenous injection of crude papain. Arch. Biochem. and Biophys. **76:** 122.
5. Tsaltas, T. 1958. Papain-induced changes in cartilage: alterations in the chemical structure of the cartilage matrix. J. Exptl. Med. **108:** 507.
6. McCluskey, R. T. & L. Thomas. 1958. The removal of cartilage matrix, *in vivo*, by

papain. Identification of crystalline papain protease as the cause of the phenomenon. J. Exptl. Med. **108:** 371.
7. Weissmann, G., J. L. Potter, R. T. McCluskey & M. Schubert. 1959. Turbidity produced by hexammine cobaltic chloride in serum of rabbits injected intravenously with papain. Proc. Soc. Exptl. Biol. Med. **102:** 584.
8. Davis, N. C. & E. L. Smith. 1955. Assay of proteolytic enzymes. Methods of Biochem. Anal. **2:** 215.
9. Kunkel, H. G. 1954. Zone electrophoresis. Methods of Biochem. Anal. **1:** 155.

BIOCHEMICAL STUDIES OF CONNECTIVE TISSUE REPAIR*

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Wound healing should be regarded as having several closely related component parts, namely, epithelization, vascularization, contraction, connective tissue repair, and the repair of specialized tissues. The repair of connective tissue is the most basic of these and appears to be related in varying degrees to the other components of repair. This study is concerned primarily with this aspect of wound healing, namely, the formation of granulation tissue. Some of the evidence to be presented is drawn from studies of connective tissue formation in situations other than in the wound itself such as the carrageenin granuloma[1,2] and the implanted polyvinyl sponge.[3]

The formation of granulation tissue has been studied extensively by histological and histochemical methods.[4-6] Several years ago these sequences were correlated by Dunphy and Udupa[7] with biochemical changes in the wound. On the basis of these studies a "normal pattern" of repair was defined. More recent studies in this laboratory and by other workers modify the picture in some respects and form the basis of the present report.

The Amorphous Matrix

One of the features of connective tissue repair is the early appearance of an amorphous matrix that stains metachromatically with methylene blue and reacts positively to stains regarded as specific for sulfated mucopolysaccharides. It has been thought that an essential component of this matrix is a rich content of mucopolysaccharides and that this material is required for the precipitation of collagen.[8,9] The concentration of hexosamine in healing wounds also has been considered supportive evidence of the presence of mucopolysaccharides.[7] Several bits of other evidence have cast doubt on this view. It is of interest that Wolbach, in his important histological studies of repair, interpreted the early wound matrix as one having the staining properties of collagen.[10] Furthermore, some years ago Kodicek and Loewi[11] pointed out that, although chondroitin sulfate can be isolated from the granulation tissue of healing tendon, the synthesis of this material as measured by the uptake of sulfate (S^{35}) does not occur until the ninth day of healing, when collagen formation is already well advanced. In our early work it was thought that the highest concentration of hexosamine in the healing wound was found between days 4 and 6, but subsequent observations with the sponge biopsy technique indicated that the peak concentration is reached immediately after wounding and falls progressively thereafter.[3] Grillo *et al.*[12] made the same observation in their studies of contraction.

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In an effort to clarify this matter, the authors have investigated the occurrence of chondroitin sulfate in granulation tissue during the healing of open and closed wounds. The distribution of bound hexosamine in the exudate obtained by the sponge biopsy technique from closed wounds also was measured.

Studies of the Wound Matrix by the Sponge Biopsy Technique

These studies were carried out on young albino male rats maintained on a standard diet. In one group of 24 animals, 10 Ivalon sponges were implanted subcutaneously, as previously described.[3] The sponges were harvested at 8 days, pooled, weighed, and divided into 2 portions.

Portion A. The sponges were homogenized and incubated with activated papain for 24 to 48 hours at 60° C. The digest was filtered through Hyflo-Super-Cel* and dialyzed against frequent changes of distilled water, and chondroitin sulfate was isolated with the use of cetyl pyridium chloride.[13]

TABLE 1

	Hexosamine (mg.)	Total hexosamine (%)	Nitrogen (mg.)	Hexosamine/ nitrogen ratio
Whole sponge	21.69*	—	3.7	0.09
Chondro. sulf.	0.48	2.2	—	—
Saline extract	17.36	80.0	175.6	0.10
Serum	0.093†	—	0.86	0.09†

* Mean of 12 sponges, each analyzed separately.
† Mean of 12 determinations on 0.1 ml.

The fraction precipitating between 0.5 and 0.9 *M* Na_2SO_4 was accepted as a polysaccharide.

Portion B. The sponges were homogenized in ice-cold 0.14 *N* NaCl and extracted in the cold exhaustively with the same solution. The extract was dialyzed, and the acid mucopolysaccharide was precipitated by the addition of cetyl pyridium chloride and then removed by centrifugation. The soluble serum-type proteins remained in the solution.

Hexosamine determinations were made on the isolated fractions by the method of Blix,[14] and nitrogen determinations were carried out on some of the fractions. Hexosamine-nitrogen ratios were also determined on samples of serum obtained just before the animals were sacrificed.

The results are tabulated in TABLE 1. Very little chondroitin sulfate was present in this tissue. Most of the bound hexosamine appeared to be associated with the extracted proteins. Moreover, the hexosamine nitrogen ratio of the unextracted sponges was almost identical with the ratio obtained in serum, indicating that the hexosamine was derived largely from serum mucoproteins. This finding is in accord with that of Noble and Boucek,[15] who found that galactosamine, the amino sugar associated with chondroitin sulfate, is not present in implanted sponges until the ninth day.

* Johns-Manville Co., New York, N. Y.

Studies of the Open Granulating Wound

Two circular patches of skin 3 cm. in diameter were removed from the back of each animal, and granulation tissue was allowed to form. The animals were divided into 6 groups of 30 each; they were sacrificed on days 6, 8, 9, 10, 12, and 15 after wounding. The scar was removed carefully and the granulation tissue dissected out carefully. The tissue was pooled and weighed. In view of the apparent importance of marginal tissue in the phenomena of contraction,[12] a narrow band of tissue 0.5 cm. in width was dissected from around the margin of the wound. Samples of skin from an unwounded area were also obtained. Chondroitin sulfate was isolated from these tissues by the technique referred to earlier.[13]

The results are shown in TABLE 2. The concentration of chondroitin sulfate in the open-wound tissue was always lower than that found in the skin. This was true throughout the entire period of the experiment. The concentration in the wound was fairly constant, although the values for the marginal tissue were somewhat higher than for the central granulation tissue itself.

TABLE 2
CONCENTRATION OF CHONDROITIN SULFATE IN GRANULATION TISSUE
In Milligrams per Gram Wet Weight

No. animals	Day	Skin		Margin		Granulation	
		Wet weight	C.S.A.*	Wet weight	C.S.A.*	Wet weight	C.S.A.*
30	6	28.5	0.66	31.3	0.44	13.6	0.23
22	8	29.3	0.60	25.0	0.39	15.9	0.16
28	9	26.5	0.71	20.4	0.42	14.8	0.20
22	10	16.1	0.73	17.6	0.45	13.7	0.28
30	12	25.0	0.63	12.4	0.48	4.2	0.30
30	15	21.9	0.71	9.6	0.40	4.8	0.29

* Chondroitin sulfate.

Insufficient material was available to identify the exact nature of the mucopolysaccharide extracted. Only chondroitin A, B, C or, possibly, keratosulfate would be expected to precipitate with cetylpyridinium chloride under the conditions used. It appears that in both instances of connective tissue formation most of the hexosamine is associated with serum mucoproteins in the wound exudate rather than with mucopolysaccharides.

From these observations it seems unlikely that a high concentration of mucopolysaccharides is an essential feature of collagen formation in the healing wound.

Collagen Formation

Biochemical studies of fibrogenesis largely confirm the histological picture, although the mechanism is seen more clearly. Collagen may be detected chemically as early as the third day after wounding, a time when no fibers are visible histologically.[2] The biosynthesis of collagen by the fibroblast and the

nature of the subsequent collagen molecule have been the subject of two recent studies.[16,17] Briefly, it appears that the fibroblast synthesizes the collagen molecule and then secretes it into the extracellular space. The first fibers probably are formed at the surface of the fibroblast. The fibers then increase in length and diameter by accretion of collagen molecules from the extracellular space. As the process continues, the constituent molecules of the fiber become aggregated more firmly as their resistance to extraction increases. By the time argyrophil fibers are visible histologically, an acid buffer is required to break up the fiber complex. With increasing maturation, the fibers become insoluble in aqueous solution and can be extracted only by the process of gelatinization. At this stage they appear as mature collagen fibers when stained by van Gieson's method. The concentration of collagen and the nature of the collagen molecule seem to parallel rather closely the increase in tensile strength of the wound.[3]

Inflammation and Granulation Tissue

There is evidence of a direct relationship between the acute inflammatory response and the subsequent reparative process.[18] The studies of Selye[19] demonstrate a specific relation between the severity and nature of an inflammatory stimulus and the subsequent healing process. Although the inflammatory process has been studied extensively by Menkin,[20] very few sequential biochemical studies of this relationship have been made. In this connection it is of particular interest that the administration of bacterial pyrogens has been described as having a favorable influence on wound healing.[21] Meier[22] has reported that cotton pledgets soaked in solutions of bacterial pyrogens evoke an increased production of granulation tissue. The effects of systemically administered bacterial polysaccharides on the formation of granulation tissue have been the subject of extensive study in this laboratory during the past year. Insufficient information is available at this time to draw any firm conclusions. As in other studies of the actions of bacterial pyrogens, there appear to be important dose-time relationships. Repeated large doses of bacterial polysaccharides seem to depress, whereas single doses given prior to wounding seem to stimulate the production of collagen. Optimal time-dose relationships thus far have not been established, but these studies indicate that this type of experimental model offers a very promising approach to a study of the relationship between inflammatory response to injury and subsequent fibroplasia.

Summary

The correlation of biochemical with histological and histochemical observations has provided a new perspective of the healing wound. It has been thought until recently that in the early stages of repair there is a high concentration of mucopolysaccharides in the wound matrix. More recent studies indicate that this is not the case. Most of the hexosamine in the wound matrix appears to be associated with serum mucoproteins. The significance of these observations and their relation to the current concepts of collagen formation have been reviewed briefly. Attention is called to a direct relation

between the initial inflammatory response and the reparative process. This is an important area for investigation by biochemical methods.

References

1. Robertson, W. V. & B. Schwartz. 1953. Ascorbic acid and formation of collagen. J. Biol. Chem. **201:** 689–696.
2. Jackson, D. S. 1957. Connective tissue growth stimulated by carrageenin: I. Formation and removal of collagen. Biochem. J. **65:** 277–284.
3. Edwards, L. C., L. N. Pernokas & J. E. Dunphy. 1957. The use of a plastic sponge to sample regenerating tissue in healing wounds. Surg. Gynecol. Obstet. **105:** 303–309.
4. Stearns, M. L. 1940. Studies on development of connective tissue in transparent chambers in rabbit's ear. Am. J. Anat. **66:** 133–176.
5. Sylven, B. 1941. Über das vorkommen von hochmolecularen esterschwefelsauren in granulationgewebe und bei der epithelregeneration. Acta chir. Scand. **86** (Suppl. 66): 10–151.
6. Penney, J. R. & B. M. Balfour. 1949. Effect of vitamin C on mucopolysaccharide production in wound healing. J. Pathol. Bacteriol. **61:** 171–178.
7. Dunphy, J. E. & K. N. Udupa. 1955. Chemical and histochemical sequences in the normal healing of wounds. New Engl. J. Med. **253:** 847–851.
8. Layton, L. L., D. R. Frankel, I. H. Sher, S. Scapa & G. Friedler. 1958. Importance of the synthesis of acidic polysaccharide for wound healing. Nature. **181:** 1543–1544.
9. Meyer, K. 1947. Biological significance of hyaluronic acid and hyaluronidase. Physiol. Revs. **27:** 335–359.
10. Wolbach, S. B. 1933. Controlled formation of collagen and reticulum. A study of the source of intercellular substance in recovery from experimental scorbutus. Am. J. Pathol. **9:** 689–701.
11. Kodicek, E. & G. Loewi. 1955. Uptake of (35S) sulfate by mucopolysaccharides of granulation tissue. Proc. Roy. Soc. London. **B144:** 100–115.
12. Grillo, H. C., G. T. Watts & J. Gross. 1958. Studies in wound healing: I. Contraction and wound contents. Ann. Surg. **148:** 145–152.
13. Scott, J. E. 1956. The preparation and fractionation of acid polysaccharides using long-chain quaternary ammonium compounds. Biochem. J. **62:** 31P.
14. Blix, G. 1948. The determination of hexosamines according to Elson and Morgan. Acta Chem. Scand. **2** (Part 1): 467–473.
15. Noble, N. L. & R. J. Boucek. 1958. Relationships of glucosamine and galactosamine to collagen formation. Federation Proc. **17:** 399.
16. Jackson, D. S. 1958. Some biochemical aspects of fibrogenesis and wound healing. New Engl. J. Med. **259:** 814–820.
17. Jackson, D. S. & J. P. Bentley. 1960. On the nature of the extractable collagens. J. Biophys. Biochem. Cytol. **7:** 37–42.
18. Edwards, L. C. & J. E. Dunphy. 1958. Medical progress. Wound healing I. Injury and normal repair. New Engl. J. Med. **259:** 224–233.
19. Selye, H. 1953. Part of inflammation in local adaptation syndrome. *In* Symposium on the Mechanism of Inflammation: at the occasion of the 19th International Physiological Congress held in Montreal Aug. 31–Sept. 4. G. Jasmin and A. Robert, Eds. 308 pp. Montreal. Acta. : 53–74.
20. Menkin, V. 1956. Biology of inflammation. Science. **123:** 527–534.
21. Olson, W. H. & H. Necheles. 1953. A study of the use of piromen^{R} for the treatment of duodenal ulcer in man. Am. J. Digestive Diseases. **20:** 372–380.
22. Meier, R. 1958. The pharmacological effects of polysaccharides. Ciba Foundation Colloquia on the Chemistry and Biology of Mucopolysaccharides. : 157–186.

CELLULAR SOURCES OF ANTIBODY: A REVIEW OF CURRENT LITERATURE*

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The complex problems involved in studying the cellular sources of antibody have given rise to a wide literature on this subject, including studies in many experimental situations, made by using a variety of techniques. Data in this area were reviewed a few years ago by McMaster[1] and by us.[2] In our review it was indicated that, following the experimental demonstration made by McMaster and his colleagues in 1935[3,4] of extractable antibody in lymph nodes, research on the cellular sources of antibody had been centered largely on the lymphatic system and that, within that system, these investigations had involved, in general, studies of the plasmacytic or lymphocytic series of cells by the following experimental approaches: correlations of cytological observations of appropriate tissues with the concentration of antibody in the serum or in extracts of the tissue examined; extraction of antibody from lymph cells; release or production of antibody *in vitro* by fragments of lymphatic organs from animals injected with antigen; aggregation of bacterial cells around tissue cells involved in antibody formation; histochemical staining for nucleic acids in lymph nodes involved in antibody formation; histochemical staining for antibody by use of the fluorescein-antibody technique; and, finally, the transfer to homologous animals of cells of lymphatic tissue.

Recent studies on the cellular basis of antibody formation have involved the continued use of some of the experimental approaches referred to above, as well as the introduction of new techniques. The studies to be reviewed below may be divided into the following groups:

(1) Studies *in situ* of antibody-forming lymphatic tissues, including (a) histochemical staining for localization of ribonucleic acid and (b) staining of lymphatic tissue with fluorescein-conjugated antibody, either antibody homologous to an antigen injected into the experimental animal or antibody to gamma globulin of the species.

(2) Studies of antibody-forming cells removed from the animal body, including (*a*) transfer of such cells to homologous recipient animals; (*b*) maintenance of such cells *in vitro* for study of antibody production; and (*c*) observations of antibody production by single cells.

Studies *in Situ* of Antibody-Forming Lymphatic Tissue

Histochemical Staining for Localization of Ribonucleic Acid

Earlier reports resulting from the applications of this technique by Ehrich *et al.*[5] and by Harris and Harris[6] were in agreement with respect to the changes in ribonucleic acid (RNA) that could be extracted from antibody-forming

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popliteal lymph nodes, but not with respect to the major localization of RNA in the cells of the node. In the report of Ehrich *et al.*,[5] the major accumulations of RNA were found in plasma cells of the medulla whereas, in the study of Harris and Harris,[6] directed chiefly at changes in the germinal centers of the cortex, early accumulations of RNA were found in the transitional forms between the reticulum cells and early members of the lymphocytic series.

Subsequently, Tanaka,[7] in experiments on rabbits with popliteal lymph nodes draining the site of an injection of cellular antigen, found RNA from the third day after the injection of antigen, most prominently in plasmablasts. These cells, large and with large and lightly stained nuclei, were identified by the finding of intermediary forms between them and the mature plasma cells. Makinodan *et al.*[8] studied the spleens of chickens that had been given a single injection of bovine serum albumin (BSA). They found a decrease in the population of cells of the lymphocytic series throughout the spleen in the first few days after the injection of the antigen, and an increase in the number of proplasmacytes to a maximum on the fourth day. The RNA content of the spleen showed only a slight increase, but this reached its peak also on the fourth day.

More recently, LaVia *et al.*[9] and Wissler *et al.*[10] studied the cellular changes in the spleen of the rat following a single intravenous injection of bacterial antigen, *Salmonella typhi*. The spleen began to increase in size on the second day, with marked mitotic activity in the red pulp. In sections stained with methyl green and pyronine, to localize deoxyribonucleic acid (DNA) and RNA, respectively, the number of pyronine-stained cells reached a maximum on the fourth day. The majority of these pyroninophilic cells appeared to have become transformed by the sixth day into small dark cells containing little cytoplasm and resembling small lymphocytes. These disappeared rapidly from the spleen, probably entering the blood stream. Few of the pyroninophilic cells were converted into mature plasma cells (far fewer than have been reported in similar situations in the rabbit).

Ward *et al.*[11] studied the histological reaction of the spleen in rabbits injected intravenously with bovine gamma globulin (BGG). They were able to circumvent the difficulty inherent in the slight immunological response of the rabbit to primary injection of protein by injecting simultaneously *Escherichia coli* endotoxin, which enhanced the production of antibody in the animals by a factor of about 5. The intensity of the histological reaction in the spleen of the rabbits was also increased by the injection of the endotoxin, without the introduction of any changes qualitatively different from those found in rabbits injected with the antigen alone.

In the spleens of these animals the lymphocytic follicles were slightly increased in size after 4 days, with an obvious increase in central reticular areas at the expense of the surrounding lymphocytic layers. Mitotic figures were increased in number in the central reticular areas of the white pulp, with marked increase in the number of the large reticular cells that contained bands of pyroninophilic cytoplasm. The degree of these changes increased up to the eighth day, at which time the white pulp was greatly enlarged and the lymphocytic follicles were increased in numbers and were at least 5 times larger than

those in the control animals. The red pulp was more cellular than that in control animals, but showed no increase in mitotic figures. In the red pulp were found small clusters of cells similar to the modified (pyroninophilic) reticular cells of the white pulp. There was no increase in the number of recognizable plasma cells in these animals.

In a subsequent study[12] Ward *et al.* examined the spleens of rabbits given second injections of BGG, 6 weeks after the first. The serum concentration of anti-BGG rose quite suddenly to a peak level by the fourth day, and was maintained there until the tenth day. The size of the follicles of the spleen, as well as the numbers of mitotic figures and pyroninophilic modified reticular cells within these follicles, was found to be roughly proportional to the antibody titer. An increase in the red pulp also was seen on days 1 and 2, the medullary cords being composed chiefly of mature medium-sized lymphocytes similar to those seen in the mantle layer of the follicles. A nonpyroninophilic cell possessing a clear vesicular nucleus with little cytoplasm was also observed. Plasma cells appeared in the red pulp of these spleens on the tenth day.

Examination of Lymphatic Tissue with Fluorescein-Antibody Conjugates

Localization of Antibody to Injected Antigen

In this experimental approach, application was made of the method described by Coons *et al.*[13] for localization of antigens in tissues by "staining" frozen sections with a solution of fluorescein-conjugated antibody. In applying this technique to the localization of antibody in tissues, it was necessary to carry out a sequence of two reactions: first, incubating the tissue section with a solution of the antigen and, second, after thus allowing combination of this antigen with antibody in the tissue cells, "staining" with the fluorescein-conjugated antibody, which by a second antigen-antibody interaction was to bring the fluorescent marker to sites where the antigen had been bound by antibody in the tissues. In the original applications of this method to the study of antibody formation, Coons and his colleagues[14] found, in animals that had received two or more injections of antigen, groups of fluorescein-stained cells in the red pulp of the spleen (following intravenous injection)[14] or in medullary cords of popliteal lymph nodes (after foot pad injection).[15] These groups of cells were identified as plasma cells by cytological study of the sections of adjacent tissue. Fluorescence of substantially lower intensity was observed occasionally in cells of the lymphoid follicles. In lymph nodes draining the site of a single injection of antigen, cells showing fluorescence appeared on the fourth day and were relatively rare, there being typically 10 to 20 such cells in a whole section. These were large cells with thin rims of faintly or moderately bright fluorescent cytoplasm scattered singly in the medullary areas near the edges of the follicles or in the medullary cords. On the sixth to eighth day the number of cells showing fluorescence had increased to about 50 or more and were mature, some of them being identified as mature plasma cells under the fluorescence microscope.

In the following study of this series, White *et al.*[16] injected protein antigens

into the feet of rabbits in a form that would enhance the antibody response. Following the injection of alum-precipitated diphtheria toxoid or ovalbumin, the draining popliteal lymph nodes were examined for antibody-containing cells by the fluorescein-antibody technique, referred to above. More fluorescein-stained cells were found in these lymph nodes than in those that were draining sites of injection of protein antigens in solution (200 to 400 isolated cells in the whole section, with 5 to 10 cell diameters between stained cells). Such cells were found in the medullary cords on the 7th day and were identified as hemocytoblasts (plasmablasts) or immature or mature plasma cells. No fluorescein-stained cells were found in the cortices of these lymph nodes.

Askonas and White[17] applied the fluorescein-antibody-staining technique to the problem of antibody formation in fragments of lymph nodes incubated *in vitro*. In this study guinea pigs were injected in the foot with ovalbumin prepared with oil adjuvant. Fragments of lymph nodes obtained from such animals 3 weeks later were found to be able to incorporate C^{14}-labeled glycine into antiovalbumin in a 4-hour period of *in vitro* incubation. The degree of such incorporation was found to correlate well with the number of antiovalbumin-containing plasma cells in the medullary cords of the nodes used for explantation, as judged by the localization of the fluorescein-antibody.

Localization of Gamma Globulin in Lymphatic Tissue

Ortega and Mellors[18] approached the problem of the possible sites of synthesis of antibody in lymphatic tissues by seeking sites of synthesis of gamma globulin with the use of the fluorescein-antibody technique. Thus in this case fluorescein was conjugated not with antibody against an antigen injected into the tissues, but rather with antibody to gamma globulin of the species under investigation. Using fluorescein-conjugated antihuman gamma globulin, these authors studied several human lymphatic tissues: spleens, lymph nodes, and lymphoid infiltrates. Evidence of synthesis of gamma globulin was found in plasma cells, both with and without Russell bodies, most of the fluorescein-stained plasma cells being found in the medullary cords of lymph nodes and in the red pulp of the spleen. Gamma globulin also was found in the germinal centers of lymph nodes and spleens. In virtually all of these centers many of the primitive reticular cells and large and medium-sized lymphocytes showed some degree of specific fluorescence. These cells, which resembled with conventional stains the other medium-sized and large lymphocytes in the germinal centers, but were distinguished from them by their fluorescein-staining for human gamma globulin, were found in aggregates so characteristic that it was considered they might be linked together to function as organized entities; they therefore were denoted as intrinsic cells of the germinal centers. The fluorescence staining for gamma globulin did not extend to the mantle of mature lymphocytes surrounding the germinal centers.

In the data obtained by these two kinds of application of the fluorescein-antibody technique two differences may be pointed out. The method involving fluorescein conjugated to antibody versus the injected antigen (see section on *Localization of Injected Antigen*, above) has the obvious advantage of specificity in that the locus of an antibody per se is identified. On the other hand,

the approach of Ortega and Mellors,[18] that of seeking sites of synthesis of gamma globulin as an indicator of possible sites of synthesis of antibodies, has the advantage of greater sensitivity. Since protein antigens are multivalent, whereas the corresponding antibodies are divalent, and since the molar-combining ratio of protein antigen to antibody is less than 1, the experimental situation in which the antigen is fixed in the tissues and the antibody is free in solution would be more sensitive than its reverse in terms of the amount of the tissue-bound reagent necessary for interaction with a given amount of the reagent in solution. This favorable combining ratio is the only one involved when antigen is being identified in tissue by fluorescein-conjugated antibody. In localizing tissue-bound antibody, however, this reaction is preceded by one in the opposite direction, with its unfavorable antibody-antigen ratio.

Studies of Lymph Node Cells Removed from the Animal Body

Transfer of Lymph Node Cells to Homologous Recipient Animals

The experimental approach of lymph node cell transfer, employed originally by Chase[19] for the study of delayed hypersensitivity, has been used widely, especially in studying the production of circulating antibody. The considerable literature in this area published before 1955 has been reviewed elsewhere.[2] It comprises studies of the transfer of cells or tissue fragments from donor animals injected with an antigen to fresh recipient animals and examination of the sera of the latter for the presence of antibody to that antigen. These earlier studies had involved several animal species (rabbits, guinea pigs, and mice); primary or secondary injections of the antigens into the donor animals; various antigens, cellular (bacterial, erythrocyte, or tumor) or soluble proteins (bacterial toxins or serum proteins, these always involving multiple injections of the donor animals); and, finally, various sources of cells, usually lymph nodes or spleens, but occasionally peritoneal exudates, lymph, thymus, etc.

Since then there has been considerable extension of the technique to studies of other animal species, additional antigens, different conditions of transfer and, especially, to the study of special problems in antibody formation.

Working with bacterial antigens, Roberts[20] gave multiple intraperitoneal injections of *S. typhi* to rabbits and then transferred fragments of the omentum of these animals by intraperitoneal injection to recipient rabbits, in the sera of which agglutinins to *S. typhi* appeared subsequently. The omenta of the donor rabbits were found to contain numerous plasma cells. Mitchison[21] transferred to young chickens the cells of spleen and bone marrow obtained from cocks that had been given repeated intravenous injections of rabbit erythrocytes. Antibody appeared in the sera of the recipients after transfer of splenic cells, but not after bone marrow transfer.

An area of special interest in the work with bacterial antigens is that of the transfer of lymph node cells incubated *in vitro* with the antigenic material. This had been demonstrated by Harris *et al.*,[22,23] who obtained lymph node cells from uninjected rabbits, incubated these *in vitro* with suspensions of whole *Shigella paradysenteriae*, washed the cells, and transferred these to irradiated recipient rabbits. Agglutinins to the *Shigella* appeared subsequently in the sera of the recipients.

The effective transfer of cells incubated *in vitro* with the source of antigenic material has been reported also in a number of other studies. Sterzl[24] incubated rabbit spleen cells with suspensions of *Salmonella paratyphi* and then transferred the mixture to young rabbits. Agglutinins to the bacterial cells appeared later in the sera of the recipients. Subsequently, he was able to demonstrate an analagous effect with suspensions of *Brucella* as the source of antigenic material.[25] Parallel observations were made by Trnka,[26] who used hen spleen cells with *S. paratyphi* and *Brucella*, and young chicks as the recipients, and by Holub,[27] who incubated rabbit lymph cells with the same antigens for transfer to newborn rabbits. Finally, Nossal[28] incubated splenic cells of mice and rats with flagellar suspensions from *Salmonella* and, on transfer of these, was able to find homologous agglutinins in the sera of recipient animals, if these were 1 week of age or older.

In further work with *Shigella*, Harris *et al.*[29] found that when rabbit lymph node cells were incubated with cell-free filtrates of trypsin-treated suspensions of *Shigella* and transferred to irradiated recipient rabbits, agglutinins to *Shigella* appeared in the sera of the rabbits. Dialysates of such filtrates also were found to contain a form of the antigen effective in *in vitro* incubation with lymph node cells.[30] By using these soluble forms of the antigenic material, it was possible to show[29] that this antigen was taken up by the cells within 5 min. of incubation at 37° C. and that during this incubation the uptake of very small amounts of the antigen (approximately 10^{-7} mg./2×10^8 cells) was sufficient for the subsequent attainment of maximal agglutinin levels in the sera of the recipients.[31]

Investigators working with protein antigens have been able to demonstrate the cell transfer effect with cells obtained from donor animals injected 2 or more times with the antigen, but less regularly and with substantially lower levels of antibody in the case of cells from donors given single injections of the antigen. Taliaferro and Talmage[32] transferred splenic cells obtained from donor rabbits 3 days after a secondary injection of these animals with BSA. On the second day after transfer of the spleen mince the antialbumin content of the sera of recipient animals was in the range of 2 to 26 μg. of antibody N per milliliter. In this study, radioactive labeling of the antibody was carried out by the administration of S^{35}-labeled amino acids to donor or recipient animals. When splenic cells were transferred from S^{35}-labeled donors to unlabeled recipients, the antibody in the latter showed very low specific radioactivity. On the other hand, the transfer of cells from unlabeled donors to labeled recipients resulted in substantially higher specific radioactivity of the antibody. These data indicated that almost all of the sulfur-containing amino acids present in the antibody were incorporated during the time of rising antibody concentration and not during the induction period. Stavitsky[33] was able to show also that the antibody found in recipients of lymph node cells from donors secondarily injected with protein antigens was synthesized from free amino acids of the recipients' tissues. Stavitsky[34] and Stavitsky *et al.*[34a] also studied the production of antibody in recipients of preparations of lymphatic tissue from donor rabbits or rats secondarily injected with protein antigens (diphtheria toxoid, BGG) or from donor animals given only primary injections of antigen, the cells in the latter case being allowed a second contact with the

antigen *in vitro* or in the recipients' tissues. In this case (lymph node cells from donors injected once with the protein antigens) it was necessary to inject into the recipient animal both antigen and cells in order to cause production of antibody.

Substantially lower levels of antiprotein antibodies have been demonstrated in recipients of cells from donors given only a primary injection of antigen. Sibal and Olson[35] injected BSA intravenously into hens and, two days thereafter, excised the spleens and obtained cell suspensions that were transplanted to the chorioallantoic membrane of embryonated eggs. The transplants were removed after 4 to 7 days of incubation, and extracts of these were found to contain low titers of antibody to BSA, as indicated by adsorption-hemagglutination tests. In experiments with guinea pigs, Rosenberg *et al.*[36] gave donor animals single intravenous injections of pooled human serum and hen egg albumin and removed the spleen and lymph nodes at various intervals thereafter. Cells obtained from these tissues were injected intradermally into recipient guinea pigs. Evidence of the development of antibody in the recipient was obtained by positive passive cutaneous anaphylaxis 24 hours or more after transfer.

Weigle and Dixon[37] found that they could obtain a primary response to BGG only when they transferred normal lymphoid cells to rabbits that had been made "tolerant" to BGG by large injections of the protein soon after birth, but not in ordinary adult irradiated rabbits (in this type of experiment both lymph node cells and antigen were injected into the recipient animal). Although 8 of 15 such "tolerant" rabbits gave an indication of primary response by antigen clearance, only 2 had significant amounts of antibody to BGG on quantitative precipitin analysis of their sera.

In the case of lymph node cells incubated *in vitro* with protein antigens, an important obstacle to obtaining a cell transfer effect may lie in the fact that the primary antibody response to protein antigens is considerably slower than that to many cellular antigens, especially those of bacterial cells. This may be of decisive importance since, as indicated below, there is evidence that the transfer of lymph node cells in a genetically heterogeneous population gives rise to a homograft reaction and, as a result, the transferred cells may be injured, in the case of the slower primary response to protein antigens, before there is time for antibody to appear. In an ingenious approach to circumventing such a difficulty, Trnka and Riha[38] injected hen spleen cells incubated *in vitro* with BSA into 18-day-old chick embryos (which presumably could not react immunologically to the antigens of the transferred cells). Several days after hatching, the chicks were bled, and antibody to BSA was detected in their sera. When such cells were transferred to newly hatched chicks, rather than to chick embryos, antibody did not appear. This difference presumably was due to the fact that, in the case of transfer to the chick embryos, tolerance to the spleen cells developed and antibody production could be demonstrated, whereas the newly hatched chicks probably rejected the spleen cells before antibody production was complete.

Before considering some special problems of lymph node cell transfer, reference should be made to the work of Jaroslow and Taliaferro,[39] who studied

the production of hemolysins to sheep erythrocytes in X-irradiated rabbits injected with this antigen, as well as with preparations of various tissues. The authors found that antibody titers of irradiated antigen-injected rabbits were increased by the concurrent injection of these animals with HeLa cell mince or extract, mince of normal rabbit spleen, or extract of normal mouse spleen. Essentially complete restoration of antibody level was effected by HeLa cell mince or extract or yeast autolysate. In all tissues that were effective in this way the induction period was about the same (6.3 to 7.6 days) as that of the irradiated rabbits (7.1 days) rather than that of the unirradiated controls (2.6 days). The restoration of antibody-forming capacity described here differs from the conventional cell transfer effect in that the transfer of whole lymph node cells leads to the appearance of antibody in the case of irradiated recipients at the same time as in that of nonirradiated recipients, the transferred cells presumably being the site of synthesis of the antibody.

Special Aspects of Lymph Node Cell Transfer

Host-tissue reactions to transferred cells. The recent developments in the study of tissue transplantation immunity have been appreciated by investigators working with the lymph node cell transfer system. Employing antibody production after transfer as an indicator of functional activity of the transferred cells, a number of workers have explored the reaction of recipients' tissue to the transferred cells. In the system involving transfer of lymph node cells incubated *in vitro* with *Shigella* antigen, Harris *et al.*[40] demonstrated experimentally that the injection of leukocytes into rabbits gave rise to an immunological reaction against the leukocytes and that in this altered host environment lymph node cells were suppressed, the antibody not appearing after transfer in the usual manner. In order to bring about this suppressive effect on the lymph node cells it was necessary to inject an adequate number of leukocytes at an appropriate time prior to the lymph node cell transfer. The suppression of lymph node cell transfer could be induced not only actively, as described above, but also passively, by the use of serum obtained from rabbits injected with leukocytes pooled from the blood of many rabbits.[41] The effect of prior injection of leukocytes on subsequent transfer also had been reflected in an experiment reported earlier by Mitchison[42] on colonization of irradiated tissue by splenic cells, in which he observed that production of antibody after transfer was impaired in host mice previously "immunized" with spleen cells of the donor strain.

Evidence of the host-tissue reaction to transferred cells was obtained in another experimental approach by Sterzl[43] and by Dixon *et al.*[44] Sterzl transferred rabbit spleen cells to young rabbits and, on succeeding days, he injected *Brucella suis* organisms. It was observed that, when the interval between cell transfer and antigen injection was as great as 4 days, antibody was produced in the recipients. When the interval was greater than 4 days there was little or no evidence of antibody production by the transferred cells. In the study of Dixon *et al.*[44] a similar experimental situation was employed. Lymph node cells from rabbits previously immunized with BSA were injected into adult irradiated recipients and, at various intervals thereafter, BSA was in-

jected. It was found that between 1 and 3 days after transfer the cells lost the ability to make a detectable response to the antigen. In both of these studies the failure of antibody to appear was attributed to the reaction of the host tissues against the cells during the period preceding the injection of the antigen.

The transfer of lymph node cells to neonatal recipients. Dixon and Weigle[45] employed the technique of cell transfer to study the nature of the immunological inadequacy of the neonatal animal. They transferred lymph node cells from donors immunized with BSA to recipients of two kinds: 5- to 6-day-old rabbits and adult irradiated rabbits. The recipients also received injections of BSA; anti-BSA was found, as expected, in sera of the adult recipients, but not in sera of the neonatal. Other experiments were carried out with a soluble antigen derived from *Shigella*. When this antigen was injected into donor rabbits and the lymph node cells of these were transferred to neonatal recipients, agglutinins to *Shigella* were found in the recipients if the injection of the antigen into the donor animals had been done 3 days before cell transfer, but not if the donors had been injected 2 hours before their lymph node cells were obtained. Adult recipients developed agglutinins in both cases. These findings led the authors to conclude that the environment provided by the neonatal recipient is unsuited to the immunological activities of transferred cells in the early phase of the immune response, but that neonatal recipients can support cells transferred during the productive phase of antibody formation. In a later study Dixon and Weigle[46] presented further data in support of this thesis by reporting that, when splenic cells from young rabbits (4 to 7 days old) were incubated *in vitro* with *Shigella* antigen and then transferred to adult irradiated recipients, agglutinins appeared in the sera of the latter (but here, again, not if the recipients were newborn). The immunological inadequacy of the neonatal rabbit therefore did not seem to hinge on the lack of cells capable of antibody formation, but rather on the tissue environment provided for these cells in the neonatal animal. Differences in response of neonatal mice of 2 strains were reported by Nossal,[28] who found that transfer of mouse spleen cells incubated *in vitro* with flagellae of *Salmonella* to newborn Hall Institute mice (1 day old or less) did not lead to the appearance of specific agglutinins whereas, after transfer to newborn mice of the C3H strain, agglutinins appeared. In 1 strain of rats tested, newborn recipients failed to develop agglutinins after transfer. For the secondary response, mice or rats of all ages were effective as recipients.

On the other hand, in a number of studies it has been found that neonatal animals of various species used as recipients could support transferred lymph node cells. Sterzl[24] reported the appearance of agglutinins after transfer of spleen cells of rabbits incubated *in vitro* with *Salmonella paratyphi* to neonatal rabbits (5 days old, an age at which Sterzl, in attempts at "active immunization," found rabbits unable to produce agglutinins to this organism). In Mitchison's[21] experiments with spleen cells of immunized cocks, young chickens (5 to 6 days old) were employed effectively as recipients, as were chickens 15 to 20 days old. Trnka[26] incubated chicken spleen cells with *S. paratyphi* and, after transfer to newly hatched chicks, detected agglutinins in the sera of the

recipients. Similar results were obtained by Holub[27] in a system involving lymph cells, *Brucella suis* as antigen, and 5-day-old rabbits as recipients; by Papermaster *et al.*,[47] with chicken spleen, *B. abortus*, and newly hatched chicks; and by Harris *et al.*,[48] with rabbit lymph node cells, soluble antigen of *Shigella paradysenteriae*, and transfer to newborn rabbits (1 day and older). Further evidence of the support of adult spleen cells in an embryonic and then neonatal environment is afforded by the work of Simonsen.[49] In this study, cells of spleen and peripheral blood of adult chickens were transferred to chick embryos on the eighteenth day of incubation. After hatching, some of the chicks developed severe hemolytic anemia, and the washed blood cells from anemic animals gave a positive direct reaction to Coombs' test. The conclusion was drawn that the cells of the transplant had formed antibody to antigens of the host tissue.

Cytological study of sites of lymph node cell transfer. In a few of the studies of transferred lymph node cells reviewed here[27,34,50] and in some of the earlier reports reviewed elsewhere,[2] cytological examinations were made of smears of the suspension of cells to be transferred. The preponderant cell types were found to be members of the lymphocytic series, their average percentage frequency being about 85 to 99, and that of small mature lymphocytes about 70 to 92. The plasma cell series was reported as accounting for 0.6 to 3 per cent of the cells, the remaining few per cent being scattered among other types of leukocytes.

In some recent studies lymph node cells have been transferred by subcutaneous or intramuscular injection, and serial histological examinations have been carried out at the site of this injection. In the first such study reported, Roberts *et al.*[50] obtained lymph node cells from donor rabbits previously injected with BSA and transferred them by intramuscular and subcutaneous injection to irradiated rabbits, which were also given BSA. In the transferred suspension of cells the average distribution was as follows: lymphocytes, 90 per cent; macrophages and reticuloendothelial cells, 8 per cent; plasma cells, 1 per cent; miscellaneous, 1 per cent. The sites of transfer of recipient rabbits were examined histologically at various intervals after transfer. After the second day there were a progressive decrease in the number of lymphocytes found in the injected tissue and the appearance of large immature pyroninophilic cells similar to those in antibody-producing spleens that were denoted by Fagraeus as "transitional cells." These cells were found in greatest numbers between the fourth and sixth days. During this period mature plasma cells appeared and reached their maximal number at the site of transfer on the eighth day. Such transitional cells and plasma cells were relatively rare when the lymph node cells were injured before transfer or were transferred without concomitant injection of antigen into the recipient. The same sequence of histological changes at the site of transfer was observed when cells of peritoneal exudates were transferred. Here the mean distribution of cells in the transferred suspension was: macrophages, 71 per cent; lymphocytes, 11 per cent; granulocytes, 16 per cent; miscellaneous, 2 per cent. The transfer of such peritoneal exudate cells was found to be of the same order of effectiveness in the production of antibody in the recipient animal.[51]

In a subsequent study Neil and Dixon[52] again observed the transition in cell population at the site of transferred lymph node cells from a predominance of lymphocytes to one of transitional cells to one of mature plasma cells. In this study, sections of the tissues also were stained for anti-BSA by the fluorescein-antibody technique. Antibody was found for the first time on the third day, chiefly in the transitional or preplasma cells. The maximal fluoresence was found on the fifth day, involving predominantly the transitional (preplasma) cells and plasma cells.

A comprehensive study of histological changes at sites of transfer of splenic and other cells, alone or with antigen, was carried out by Holub[53] in various experiments, including autotransplantation and homotransplantation. In the latter, the transfer of splenic cells was carried out with and without the concurrent injection of antigen. Autotransplantation of splenic cells intraperitoneally caused the appearance of small necrotic centers with various leukocytes, surrounded by a gelatinous capsule, below which there was marked proliferation of reticulum cells. In 2-month-old rabbits so treated, groups of typical mature plasma cells always were found. Intraperitoneal homotransplantation of lymphoid cells without antigen caused the appearance of macrophages and more mature lymphocytes and, within 6 to 8 days, of transitional pyroninophilic cells that then developed into plasma cells. All these cells were considered to be the result of differentiation of the transferred cells. Subcutaneous homotransplantation of living lymphoid cells without antigen led to the same results, except that the number of plasma cells was smaller. The injection of lymphoid cells with antigen (*Brucella suis*, *Salmonella paratyphi*) caused similar histological changes at the site of transfer. Thus, in this series of experiments, maturation of the plasma cell series at the site was observed whether or not an antigen also was injected, and without evident relationship to the degree of antibody response to the injected antigen. Holub concluded from these studies that "lymphocytes, as well as primitive reticular or primitive mesenchymal cells, appear to enter the inductive phase of antibody formation, while the productive phase is associated with plasma cell differentiation." In the case of the differentiation of cells injected without accompanying bacterial antigen, Holub suggested that the antigens of the host tissue provided the stimulus, as part of a homograft-versus-host reaction.

Production of Antibody *in Vitro*

There have been a number of recent studies of antibody production *in vitro* by lymph node or splenic tissue. The majority of these studies have been carried out with tissues obtained from animals injected with the antigen, as in earlier studies in this area.

Stavitsky[54] injected rabbits twice with diphtheria toxoid and, 3 days after the second injection, excised the draining lymph nodes and spleen. Fragments of these tissues were incubated in Fischer's medium with 20 per cent normal rabbit serum at 37° C. for 1 day, and the medium then was tested for antitoxin, usually by adsorption-hemagglutination. Antitoxin was found in such preparations in greater amount than could be extracted from the explanted tissue or than appeared in control preparations. It was estimated that the

antibody produced *in vitro* per unit of explanted lymph node or spleen was approximately one tenth the amount that would have been produced by the same amount of tissue on transfer to recipient rabbits.

Later Wolf and Stavitsky[55] were able to produce antitoxin in such tissues maintained in synthetic medium (Tyrodes' solution, fructose diphosphate, and an amino acid mixture). The amount of antitoxin produced in this synthetic medium per milligram of tissue was generally less than that in the Fischer's medium with rabbit serum, although the addition of rabbit serum to the synthetic medium did not increase the antibody production. Using this medium, they were able to show that the synthesis *in vitro* of diphtheria antitoxin by fragments of lymph node from toxoid-injected rabbits could be inhibited by the use of amino acid analogues, this inhibition being reversible by further addition of the amino acids.[56]

Splenic cell suspensions were obtained by Steiner and Anker[57] from rabbits twice injected with BSA. The cells were maintained *in vitro* in suitable media in an incubation vessel consisting of a horizontal cellophane membrane supporting a layer of spleen cells that had been deposited as a suspension and forming the top of a reservoir of medium that could be changed during the experiment. When splenic cells obtained from rabbits 3 days after the secondary injection with BSA were incubated in this vessel, anti-BSA could be detected subsequently in the medium above the cellophane membrane. The concentration of anti-BSA reached a maximum in about 2 days. It was calculated that the antibody per cell produced under these conditions was nearly the amount that would be produced on transfer of such spleen cells to a homologous recipient. However, less antibody was produced when the spleen cell suspension was prepared from spleens excised 2 days after the secondary injection of BSA into the rabbit, and none if the spleen was removed 1 day after that injection.

Similar time relations in experiments on explantation of splenic cells were observed by Sterzl,[58] who obtained such cells from rabbits injected with *Brucella* and maintained them *in vitro*. Agglutinins to *Brucella* were found often in the tissue culture fluids 2 days after explantation, but only if the splenic cells were obtained not less than 48 hours after the injection of the antigen. Cells mixed with antigen *in vitro* and then maintained in tissue culture did not produce antibody.

More recently, Grabar and Corvazier[59] obtained lymph nodes or spleens from rabbits or chicks that had been given primary injections of *Salmonella paratyphi* or human serum albumin, and incubated fragments of these in agar gel on screens of stainless steel kept in rotating tubes. Homologous antibodies were found during the next 4 to 6 days in the case of the splenic explants, and the next 6 to 8 days in the case of lymph node tissue, the response being relatively feeble in the case of the protein antigen. Such antibodies appeared only when the tissues were obtained 3 days or more after the animal had been injected with antigen.

Another approach to the study of *in vitro* antibody production by lymphatic tissues was taken by Askonas and Humphrey,[60] who incubated tissues from animals injected with ovalbumin in a medium containing C^{14}-labeled amino acids. They observed the degree of incorporation of the labeled amino acids

into antiovalbumin during the first few hours of incubation. Rabbits that had received intramuscular injections of ovalbumin in an oil-adjuvant mixture showed most activity in the slices of local granuloma tissue but, after further intravenous injection of alum-precipitated antigen, the explants of spleen, lung, and bone marrow showed more activity. In rabbits injected simultaneously with two different antigens, ovalbumin and killed pneumococci, differences were found in the production of each kind of antibody by explants of various tissues. The tissues showing the greatest degree of activity in this study were those that had been found by Askonas *et al.*[61] to be active in incorporating labeled amino acids into gamma globulin: spleen, lymph nodes, and bone marrow.

In two recent studies the production of antibody *in vitro* has been reported in the case of tissues obtained from animals not injected with the antigen. Stevens and McKenna[62] used splenic tissue of rabbits injected with *Salmonella typhi* endotoxin. Such tissue was diced, incubated for 1 hour at 37° C. with a solution of BGG, and then incubated further at that temperature in a medium similar to that of Trowell. Culture medium was withdrawn at intervals and tested for anti-BGG activity by the adsorption-hemagglutination technique. Titers of the order of 200 were obtained in such fluids after 1 day, barely detectable titers being observed after even 1 hour of incubation. In some experiments the splenic tissue was obtained from rabbits previously injected with BGG. When such tissue was incubated *in vitro* with this antigen and explanted, anti-BGG hemagglutination was observed in the same range of titer as in the case of splenic tissues obtained from endotoxin-injected rabbits and given primary contact with BGG *in vitro*. The conclusion that the hemagglutinating activity in the culture fluids was due to anti-BGG antibody was supported by the observations that it was not produced by non-BGG-incubated tissue (for example, splenic tissue incubated with casein), that the hemagglutination reaction was inhibited by BGG in solution, and that complement fixation was obtained at correspondingly lower titers of the fluid. On the other hand, for the interpretation of these results it should be pointed out that it was necessary to keep the fluid frozen at −40° C. to avoid erratic results in the titrations, and that it was necessary to incubate the explanted tissue with BGG at a concentration of 0.5 to 5 mg./ml. to obtain the results described.

The other report of antibody formation *in vitro* by tissues of nonantigen-injected animals is that of Fishman,[63] who incubated rat lymph node cells *in vitro* with antigens (hemocyanin, bacteriophage T2) that had been prepared by incubation for 30 min. at 37° C. with a suspension of peritoneal exudate cells, largely macrophages. Tissue culture fluids collected at various days were tested by adsorption-hemagglutination in the case of the hemocyanin-tested cells, and by neutralization in the bacteriophage experiments. Hemagglutinating and neutralizing substances, respectively, appeared in barely detectable concentrations on the fifth and seventh days in the respective cultures. Thereafter, the relative increases to the eleventh day were considerable, but even then the titers were rather low (for example, hemagglutination titer of 160). The interpretation of these results should await confirmation or am-

plification of the data, in view of the low final concentration of hemagglutinating or neutralizing substances and, especially, because of the considerable length of time during which the concentration of these substances increased in a culture of cells that, in the case of the rabbit at least, are notably short-lived.

Antibody Formation by Single Cells

Recently several investigators have undertaken the study of the production of antibody by single cells, these studies being directed primarily at the question of whether a single cell can form antibodies to more than one antigen.

Nossal and Lederberg[64] and Nossal[65] gave rats repeated injections of a mixture of two immunologically distinct types of *Salmonella* and then prepared suspensions of cells from the draining lymph nodes. Microdroplets of the suspension were deposited on a cover slip under oil and, after 4 hours of incubation at 37° C., a suspension of about 10 motile bacteria of one of the types used for immunization was added to each droplet. Immobilization of all the added bacteria (in about 20 min. at room temperature) was taken as evidence of the presence of the homologous antibody. In the case of droplets containing one cell that caused the immobilization of bacteria of one strain, a suspension of the same number of motile bacteria of the other strain was added. In no case did a single-cell droplet give evidence of containing antibody to the flagellar antigens of both bacterial types, that is, no such droplet caused the immobilization of all the bacteria of the second type after causing immobilization of all the bacteria of the first. Subsequently, Nossal[66] applied these techniques to lymph node cells of rats given single injections of suspensions of flagellae prepared from one of the same strains of *Salmonella*. Cells were found that produced antibody, as adjudged by the criterion described above, but these comprised only 2.3 per cent of the single-cell droplets tested, in contrast to approximately 14 per cent in the case of suspensions from secondarily injected animals.

Antibody production by single cells was studied *in situ* by White,[67] who used fluorescein-conjugated antibody. Two experimental approaches were used: successive "stainings" with two different fluorescein-conjugated antibodies (with quenching of the fluorescence of the slide between stainings) and the use of red and green fluorochromes (rhodamine and fluorescein isocyanate) for conjugation with the respective antibodies. The spleen was studied after multiple intravenous injections of pneumococci and ovalbumin, and popliteal lymph nodes were studied after secondary injections of diphtheria toxoid and egg albumin into the foot. Considerable proliferation of plasma cells and their precursors was found in the pulp of the spleens and in medullas of the lymph nodes. Fluorescence was found in clumps of plasma cells in these areas, but staining with the two different fluorescein-antibody conjugates was not observed in the same groups of cells. The production by one cell of two antibodies was thus not indicated, although the possibility could not be excluded that the two antibodies were present in a single cell in widely differing amounts.

The production of antibodies to bacteriophage by single cells removed from the animal body has been studied by Attardi *et al.*[68] In this study, repeated injections of bacteriophages T2 and T5 were made in the feet of rabbits. Then

the popliteal lymph nodes were removed, cell suspensions were prepared, and microdrops of these were incubated under oil with mixtures of T2 and T5 bacteriophage. After 48 hours of such incubation, antibodies to T2 and T5 were sought in such droplets, and the concentration of such antibody was estimated by diluting the contents of a droplet with broth and then plating with the indicator bacteria. Among the single-cell droplets examined in this way, some (approximately 10 per cent) were found to contain antibody to T2 or T5 bacteriophage. A number of the single-cell droplets was found to contain antibody to both bacteriophages. In control experiments it was found that mixtures of cells obtained from rabbits, each of which had been hyperimmunized with either T2 or T5, but not with both, yielded no single-cell droplets with activity against both bacteriophages.

The conclusion of this study, that a single cell can form antibody to more than one antigen, differs from that of Nossal[65] in the comparable experiments described above. Quantitative considerations suggest that the conclusions of Attardi *et al.*[68] are the more likely to be valid. First, the measurements of neutralizing antibodies to bacteriophage were made on an essentially continuous scale, rather than from a threshold of bacterial immobilization (immobilization of *all* the bacteria in the test suspension being the sole acceptable indication of antibody). Second, the measurements of antibacteriophage antibodies were made in a far wider range of the minimally detectable units of antibody than were the estimations of immobilizing antibodies. Thus, in the latter system, when single-cell droplets from rats injected with 1 strain of *Salmonella* were tested repeatedly with portions of the bacterial suspension, only 5 of 15 such drops showed immobilization of a second lot of bacteria, indicating that many of the cells produced little more than 1 unit of antibody as defined in these studies. In contrast, the bacteriophage inactivation experiments showed a ratio between 20 and 50 of mean background (control) level of plaque counts and plaque counts of antibody-containing droplets. At the present writing, therefore, it appears that the antibody-producing cells probably can produce more than one type of antibody.

These studies are of particular interest in view of the currently debated hypothesis of the selective theory of antibody formation put forth by Jerne[69] and elaborated with additional aspects of clonal selection by Talmage,[70] Burnet,[71] and Lederberg.[72] The acceptance of the conclusion that one cell may be the site of synthesis of more than one type of antibody poses substantial difficulties for the hypothesis of clonal selection and, as pointed out by Cohn, the tenability of this hypothesis would weaken very rapidly with increases in the number of types of antibody attributed to one antibody-forming cell.

Histological Observations in the Studies of Antibody Formation by Single Cells in Vitro

In both of these investigations cytological observations were made on the cells in antibody-containing droplets. In a study by Nossal,[73] almost all of the antibody-producing cells were identified, by staining with orcein and light green, as plasma cells, whether these cells were obtained after primary or after secondary stimulation of the lymph node. In all of 93 cells in single-cell

antibody-containing droplets 91 were of the plasmacytic series. On the other hand, in the study by Attardi *et al.*,[68] cell types of both the lymphocytic and plasmacytic series were found to be associated with antibody formation. Thus, in the microdrop experiments, 11 per cent of cells of the lymphocytic series and 32 per cent of cells of the plasmacytic series produced antibody and, in the analogous micropipet experiments on the single-cell preparations, 14 per cent of cells of the lymphocytic series and 27 per cent of cells of the plasmacytic series were found in antibody-containing preparations.

Summary

The chief emphases or advances in studies of the past half decade on the cellular sources of antibodies have been concentrated in a few areas of investigation, and the current status of each of these areas may be summarized very briefly as follows:

First, in the *histological* and *cytological* areas, the problems of the previous decade on the role of the major parts of lymphatic organs and of cell types within those organs in synthesizing antibody have not been solved yet, as indicated by the data reviewed above. Thus a number of the recent studies, chiefly those of Coons,[14] White,[16] Askonas,[17] and their collaborators, indicate that the red pulp of the spleen or medullary cores of lymph nodes are the primary sites of antibody and that the plasmacytic series are the cells involved in the synthesis. On the other hand, in the study of Ward *et al.*,[11] the white pulp of the spleen, particularly the lymphocytic follicles, appeared to be involved in the production of antiovalbumin in the rabbit. Further, Ortega and Mellors[18] localized gamma globulin in (1) plasma cells of splenic red pulp and medullary cords of lymph nodes and in (2) medium and large lymphocytes of germinal centers of lymph nodes and spleens. Again, in investigations on antibody formation by single cells *in vitro*, the data of one study[73] indicated that virtually all the antibody-forming cells were of the plasmacytic series, but in another study a fraction of such cells was found to be of the lymphocytic series.[69]

One type of observation that might suggest a role in antibody formation for cells of both the lymphocytic and plasmacytic series is that of Dixon and his collaborators[51] who, as indicated above, reported the presence of plasma cells in tissues into which homologous lymph node cells had been transferred. Thus a scheme is suggested according to which the induction of antibody formation can take place in a cell recognized as a lymphocyte, and the synthesis of the antibody can take place after the transition of this cell into a type recognized as a plasma cell. The results of Dixon and his collaborators[51] indicated a constant relation between antibody formation and the transition of the transferred cells to plasma cells, whereas it was found subsequently in parallel studies of Holub[53] that cells of the plasmacytic series matured at the site of lymph node cell transfer, whether or not antigen was also injected. However, Holub suggests the possibility that, in the case of transfer of cells without antigen, a graft-versus-host tissue reaction accounts for the presence of the plasma cells.

In studies of *homotransfer of lymph node cells*, the areas of greatest activity in the past few years have been concerned with the following: extension of the

technique of cell transfer to additional species and antigens; stimulation of transferred cells *in vitro* by some bacterial antigens; study of cells found at the site of transferred lymph node cells, with the possible implications of participation in different phases of antibody formation of cells identified as lymphocytes and plasma cells; recognition of a "homograft-type" reaction against transferred cells by tissues of recipient animals and exploration of some of this reaction on the results of cell transfer; and study of some aspects of the host-tissue environment in relation to the effectiveness of transferred cells (neonatal versus adult tissues).

In the area of *in vitro* synthesis of antibody there has been recent study of the conditions for synthesis of antibody by lymphatic tissue of animals injected with antigen. Furthermore, new evidence that antibody can be synthesized *in vitro* by such tissues has been obtained by the demonstration of incorporation of radioactive amino acids of the medium into antibody and by the inhibition of such synthesis by the addition of amino acid analogues in the medium. As to the question of induction as well as synthesis of antibody *in vitro*, two studies have been reported, but the interpretation of these should await confirmation and extension of the data.

Finally, single cells from appropriately selected lymphatic tissue of antigen-injected donors have been found to produce detectable amounts of antibody. These recent demonstrations are of considerable interest, both intrinsically and because of the experimental studies made possible by this development, such as critical examination of the newly promulgated clonal or selective hypothesis of antibody formation.

References

1. McMaster, P. D. 1953. *In* The Nature and Significance of the Antibody Response. A. M. Pappenheimer, Jr., Ed. Columbia Univ. Press. New York, N. Y.
2. Harris, T. N. & S. Harris. 1956. Am. J. Med. **20:** 114.
3. McMaster, P. D. & S. S. Hudack. 1935. J. Exptl. Med. **61:** 783.
4. McMaster, P. D. & J. G. Kidd. 1937. J. Exptl. Med. **66:** 73.
5. Ehrich, W. E., D. L. Drabkin & C. Forman. 1949. J. Exptl. Med. **90:** 157.
6. Harris, T. N. & S. Harris. 1949. J. Exptl. Med. **90:** 169.
7. Tanaka, N. 1953. Japan. J. Exptl. Med. **23:** 89.
8. Makinodan, T., R. F. Ruth & H. R. Wolfe. 1954. J. Immunol. **72:** 45.
9. LaVia, M. F., F. W. Fitch, C. Gunderson & R. W. Wissler. 1957. RES Bull. **3:** 15.
10. Wissler, R. W., F. W. Fitch, M. F. LaVia & G. H. Gunderson. 1957. J. Cellular Comp. Physiol. **50:** 265.
11. Ward, P. A., A. G. Johnson & M. R. Abell. 1959. J. Exptl. Med. **109:** 463.
12. Ward, P. A., A. G. Johnson & M. R. Abell. 1960. Fed. Proc. **19:** 199.
13. Coons, A. H., H. J. Creech, R. N. Jones & E. Berliner. 1942. J. Immunol. **45:** 732.
14. Coons, A. H., E. H. Leduc & J. M. Connolly. 1955. J. Exptl. Med. **102:** 49.
15. Leduc, E. H., A. H. Coons & J. M. Connolly. 1955. J. Exptl. Med. **102:** 61.
16. White, R. G., A. H. Coons & J. M. Connolly. 1955. J. Exptl. Med. **102:** 73.
17. Askonas, B. A. & R. G. White. 1956. Brit. J. Exptl. Pathol. **37:** 61.
18. Ortega, L. G. & R. C. Mellors. 1957. J. Exptl. Med. **106:** 627.
19. Chase, M. W. 1945. Proc. Soc. Exptl. Biol. Med. **59:** 134.
20. Roberts, K. B. 1955. Brit. J. Exptl. Pathol. **36:** 357.
21. Mitchison, N. A. 1957. Folia Biol. (Praha) **3:** 72.
22. Harris, T. N., S. Harris & M. B. Farber. 1954. Proc. Soc. Exptl. Biol. Med. **86:** 549.
23. Harris, S. & T. N. Harris. 1955. J. Immunol. **74:** 318.
24. Sterzl, J. 1957. Folia Biol. (Praha). **3:** 1.
25. Sterzl, J. 1958. Transplantation Bull. **5:** 73.

26. Trnka, Z. 1957. Česk. Mikrobiol. **2:** 344.
26*a*. Trnka, Z. 1958. Transplantation Bull. **5:** 73.
27. Holub, M. 1958. Nature. **181:** 122.
28. Nossal, G. J. V. 1959. Immunology. **2:** 137.
29. Harris, S., T. N. Harris, C. A. Ogburn & M. B. Farber. 1956. J. Exptl. Med. **104:** 645.
30. Harris, T. N., S. Harris, C. A. Ogburn & M. B. Farber. 1958. J. Immunol. **80:** 308.
31. Harris, S. & T. N. Harris. 1958. J. Immunol. **80:** 316.
32. Taliaferro, W. H. & D. W. Talmage. 1955. J. Infectious Diseases. **97:** 88.
33. Stavitsky, A. B. 1958. Brit. J. Exptl. Pathol. **39:** 46.
34. Stavitsky, A. B. 1957. J. Immunol. **79:** 187.
34a. Stavitsky, A. B., A. E. Axelrod & J. Pruzansky. 1957. J. Immunol. **79:** 200.
35. Sibal, L. R. & V. H. Olson. 1958. Proc. Soc. Exptl. Biol. Med. **97:** 575.
36. Rosenberg, L. T., M. H. Chandler, A. S. Gordon & E. E. Fischel. 1958. J. Immunol. **81:** 136.
37. Weigle, W. O. & F. J. Dixon. 1959. J. Immunol. **82:** 516.
38. Trnka, Z. & I. Riha. 1959. Nature. **183:** 546.
39. Jaroslow, B. N. & W. H. Taliaferro. 1956. J. Infectious Diseases. **98:** 75.
40. Harris, T. N., S. Harris & M. B. Farber. 1958. J. Exptl. Med. **108:** 21.
41. Harris, S., T. N. Harris & M. B. Farber. 1958. J. Exptl. Med. **108:** 411.
42. Mitchison, N. A. 1956. Brit. J. Exptl. Pathol. **37:** 329.
43. Sterzl, J. 1958. Československ. mikrobiol. **3:** 61.
44. Dixon, F. J., W. O. Weigle & M. P. Deichmiller. 1959. J. Immunol. **82:** 248.
45. Dixon, F. J. & W. O. Weigle. 1957. J. Exptl. Med. **105:** 75.
46. Dixon, F. J. & W. O. Weigle. 1959. J. Exptl. Med. **110:** 139.
47. Papermaster, B. W., S. G. Bradley, D. W. Watson & R. A. Good. 1959. Proc. Soc. Exptl. Biol. Med. **102:** 260.
48. Harris, T. N., S. Harris & M. B. Farber. 1959. Proc. Soc. Exptl. Biol. Med. **102:** 495.
49. Simonsen, M. 1957. Acta Pathol. Microbiol. Scand. **40:** 480.
50. Roberts, J. C., F. J. Dixon & W. O. Weigle. 1957. A. M. A. Arch. Pathol. **64:** 324.
51. Dixon, F. J., W. O. Weigle & J. C. Roberts. 1957. J. Immunol. **78:** 56.
52. Neil, A. L. & F. J. Dixon. 1959. A. M. A. Arch. Pathol. **67:** 643.
53. Holub, M. 1959. Folia Microbiol. **4:** 137.
54. Stavitsky, A. B. 1955. J. Immunol. **75:** 214.
55. Wolf, B. & A. B. Stavitsky. 1958. J. Immunol. **81:** 404.
56. Stavitsky, A. B. & B. Wolf. 1958. Biochem. Biophys. Acta. **27:** 4.
57. Steiner, D. F. & H. S. Anker. 1956. Proc. Natl. Acad. Sci. **42:** 580.
58. Sterzl, A. 1959. Folia Microbiol. 4 : 91.
59. Grabar, P. & R. Corvazier. Ciba Foundation Symposium: Cellular Aspects of Immunity. London, England. In press.
60. Askonas, B. A. & J. H. Humphrey. 1958. Biochem. J. **68:** 252.
61. Askonas, B. A., J. H. Humphrey & R. R. Porter. 1956. Biochem. J. **63:** 412.
62. Stevens, K. M. & J. M. McKenna. 1958. J. Exptl. Med. **107:** 537.
63. Fishman, M. 1959. Nature. **183:** 1200.
64. Nossal, G. J. V. & J. Lederberg. 1958. Nature. **181:** 1419.
65. Nossal, G. J. V. 1958. Brit. J. Exptl. Pathol. **39:** 544.
66. Nossal, G. J. V. 1959. Brit. J. Exptl. Pathol. **49:** 118.
67. White, R. G. 1958. Nature. **182:** 1383.
68. Attardi, G., M. Cohn, K. Horibata & E. S. Lennox. 1959. Bacteriol. Rev. **23:** 213.
69. Jerne, N. K. 1955. Proc. Natl. Acad. Sci. U. S. **41:** 849.
70. Talmage, D. W. 1959. Science. **129:** 1643.
71. Burnet, F. M. 1957. Australian J. Sci. **20:** 67.
72. Lederberg, J. 1959. Science. **129:** 1649.
73. Nossal, G. J. V. 1959. Brit. J. Exptl. Pathol. **40:** 301.

HIGH MOLECULAR WEIGHT ANTIBODIES

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Despite the fact that the existence of high molecular weight antibodies has been known for a long time, only recently have they received attention commensurate with their significance. Several explanations may be cited for this fact. First and perhaps foremost are the fallacious concepts that these antibodies occur in only a few species (particularly the horse) and that the rabbit and the human produce only low molecular weight antibodies in response to antigenic stimulus. This was based on early work with antipneumococcal antibodies, in which few animals were examined. It now appears that not only do many horses make low molecular weight antibodies but that rabbits can make the high molecular weight type, even to pneumococci.[1] Of essential importance are the stage of immunization and variations among individual animals.

Another factor hindering their recognition was the belief that high molecular weight antibodies were artifacts produced by the aggregation of γ globulin under chemical treatment. The failure of Cann[2] to find this species in human γ globulin isolated by electrophoresis convection contributed significantly to this concept. It is now clear that γ globulin isolated by other methods of electrophoresis does contain a high molecular weight component, representing as much as 10 per cent of the total γ globulin.[3]

The high molecular weight material is a clearly definable entity in human and rabbit serum, with physical and chemical properties totally different from ordinary γ globulin. Antibodies belonging to this class also show these very different properties. It is no longer possible to say that a given material is or is not an antibody on the basis of its properties; the class of antibody, whether the ordinary 7S antibody or high molecular weight type, must be considered. In addition to these, a third group of "immunoglobulins," recently defined by Heremans, contains antibodies of still different properties, the β_{2A} proteins.[4] There undoubtedly are further subgroups.

Before discussing the various special characteristics of the high molecular weight immunoglobulins, it might be well to consider the various antibodies and other materials from human serum that have been classified in this fraction.

A summary of the types of antibodies and antibodylike substances of human serum that have been found to belong, at least in part, to the high molecular weight class is given in TABLE 1. Of course, the prototype of these proteins is the Waldenström macroglobulins. Some of these appear to have specific antibodylike activity. Cold agglutinin activity[5] and complement fixation activity with human cellular antigens[6] have been described. In addition, we have recently encountered two cases of typical Waldenström macroglobulinemia in which the pathological proteins had rheumatoid factor activity. One of these[7] precipitates at very high dilutions with aggregated γ globulin. It also gives some of the other rheumatoid factor reactions, such as those of the Rh-sensitized human cell and the Fr. II chromium-treated red cell. It does

not give all of the latex fixation test reactions nor the sensitized sheep cell test reaction. The behavior is different from that of rheumatoid factors in patients with rheumatoid arthritis, although there are many similarities.

An attractive current hypothesis is that the Waldenström macroglobulins represent the products of antibody-producing cells slightly altered through somatic mutation, but with the pathological protein still possessing some of the specificity of the antibody-producing clone from which it was derived.[8]

The rheumatoid factors are all of the 19S class and show specificity for combining with ordinary γ globulin or aggregated γ globulin.[9,10] Evidence is

Table 1

19S Class Substances in Human Serum

1. Waldenström macroglobulin	7. Lupus factors*
2. Rheumatoid factors	8. Isoagglutinins*
3. Properdin	9. Sal. Rh agglutinins
4. Conglutinin*	10. Leukoagglutinins
5. Cold agglutinins	11. Thyroid autoantibodies*
Nat., atyp. pn.	12. Typhoid-paratyphoid agglutinins*
Acq. hem. an.	13. Wassermann antibodies*
6. Heterophil antibodies	
Nat., inf. mono.	

* Partially in 7S class.

Table 2*

Protein fraction	L.E. cell activ.	Heterophil titer	
		Case 1	Case 2
1. Album.	+	0	0
2. 7S	++++	0	0
3.	+	1/16	1/4
4. 19S	0	1/512	1/64

* Localization of the heterophil antibody from the serum of 2 cases of infectious mononucleosis in the bottom (19S) fraction following density gradient zone ultracentrifugation. L.E. cell activity separated in the same experiment localized in the 7S fraction.

accumulating that they may well be anti-γ globulin antibodies. Recently there is some indication that a 7S counterpart of the 19S rheumatoid factors occurs in certain sera.[11] This forms a wide assortment of γ globulin complexes. Properdin has been reported to be a 19S class protein,[12] although some controversy remains on this point. Bovine conglutinin does not appear to be of high molecular weight, but many of the immunoconglutinins fall into this class. Recent observations in collaboration with R. R. A. Coombs (unpublished observations) indicate considerable variation from one serum to another regarding the immunoconglutinins.

The cold agglutinins all appear to be of the 19S class.[2,5,10,13] These are probably the most widely studied of the human 19S proteins that appear to have antibody properties. The observations of Wiener *et al.*[14] on I specificity for the cold agglutinins have recently been confirmed in several laboratories.

The heterophil antibodies in the serum of patients with infectious mononucleosis also appear to be entirely of this class. TABLE 2 shows results obtained with one such serum by density gradient ultracentrifugation.[5] A lupus serum with lupus erythematosis (L.E.) cell activity in the 7S γ globulin was separated at the same time for comparison. All of the heterophil antibodies were found at the bottom of the tube in the 19S fraction. Another serum from a different patient showed identical results, as did two sera from patients with liver disease with heterophil antibodies of a different type.

TABLE 3*

Protein fraction	L.E.		Sjögren's syndr.	Biliary cirrhosis
	Case 1	Case 2		
1. Album.	0	0	0	0
2. 7S	32	64	512	4
3.	32	4	64	8
4. 19S	256	0	0	256

* Localization of anticytoplasmic "antibodies" in different fractions of 4 positive sera by density gradient centrifugation. One of the L.E. sera and the biliary cirrhosis serum showed the activity in the bottom (19S) fraction. The L.E. serum and that from Sjögren's syndrome showed the activity localized in the 7S fraction.

TABLE 4
SALINE ANTI-A ISOAGGLUTININ FRACTIONATION PATTERNS (IN TITERS)

Fraction	Type 1 (19S pattern)					Type 2 (7S pattern)					Type 3 (mixed)				
	5	10	20	40	80	5	10	20	40	80	5	10	20	40	80
I	0	0	0	0	0	++	+	0	0	0	++	+	0	0	0
II	±	0	0	0	0	+++*	++*	+	±	0	+++*	++*	+	+	0
III	+	0	0	0	0	++	±	0	0	0	++	+	0	0	0
IV	++++	++†	+†	+	±	0	0	0	0	0	+++†	++	+	+	0
V	+	±	0	0	0	0	0	0	0	0	+†	+	0	0	0

* Fraction 7S.
† Fraction 19S.

Certain of the lupus factors also appear to be of the 19S class, although the L.E. cell factor clearly is of low molecular weight, as indicated in TABLE 2. TABLE 3 shows the density gradient results with complement fixation reactions of tests utilizing cytoplasmic antigens and sera from patients with lupus erythematosus, Sjögren's syndrome, and biliary cirrhosis, the three conditions giving the most positive results. These are published in greater detail elsewhere.[15] Similar results also have been obtained with chromatographic methods.[16]

Many of the isoagglutinins are of the high molecular weight class, but there is striking variation from one serum to another, and a few sera show only low molecular weight isoagglutinins. TABLE 4 shows the three types of pattern found in different sera for the anti-A isoagglutinins. The presence of both types in the same serum was not uncommon. These results also were obtained

by density gradient ultracentrifugation, and show the position of the activity at different levels from the top to the bottom of the centrifuge tube. Many of the rarer types of isoagglutinins are primarily of the 19S class.[17]

The typhoid H and O agglutinins may be of either 7S or 19S class, and also vary from one serum to another (R. Smith, personal communications). The Wassermann antibodies also appear to be of both classes.[20] The vast majority of substances belonging to the high molecular class have low molecular weight counterparts, although the latter may not be as readily detectable.

Specific Properties of 19S Class Substances

Sedimentation properties. All the very different substances illustrated in TABLE 1 and belonging to the 19S class of proteins appear to have very similar sedimentation constants for the major active constituent. This ranges from 18 to 20S. With those sera where high concentrations are present, work on mixing these proteins has been carried out. Such mixtures for the Waldenström macroglobulins, cold agglutinins, and rheumatoid factors do not resolve into separate components in the analytic ultracentrifuge. Occasionally, slight broadening of the 19S peak occurs, but usually a single sharp peak continues to be present.

Besides showing the main 19S peak, these proteins always appear to be associated with subfractions of even higher sedimentation rate. In the sera of patients with Waldenström macroglobulinemia, a 29S component and a 38S component make up 17 and 5 per cent of the total macroglobulin content, respectively. The ratio of these components was found to be surprisingly constant.[19] They have been observed clearly in all the Waldenström macroglobulins, the cold agglutinins, and the rheumatoid factors, that is, in those instances where high concentrations of 19S proteins have been obtained.

Electrophoretic mobility. Most of the proteins of this class have an electrophoretic mobility at *p*H 8.6 that is fast compared to the main portion of γ globulin. In general, they migrate in the valley between the γ and β globulin. However, their distribution is broad, and in different instances individual members of the group may be primarily in the γ peak or, rarely, in the β zone. The isoelectric point of these proteins is even lower than their mobility at *p*H 8.6 would indicate: usually between 6 and 6.5. Antibodies of rapid mobility should be suspected of being in the 19S class.

Antigenic properties. Although there is some cross reaction between ordinary 7S γ globulin and the 19S class, they differ considerably in antigenic properties.[20] The 19S material possesses antigenic determinants not found in the 7S proteins, and the reverse is also true. In Ouchterlony plate studies, the 19S proteins are distinguished by the curvature of the line toward the antigen well, caused by the slow diffusion of these molecules.[21] This often makes it possible to recognize this constituent in a mixture. In immunoelectrophoretic studies, it has been given the name β_{2M}, and the European literature utilizes this term to characterize the entire 19S class of proteins.[22]

High carbohydrate content. One of the most striking characteristics of the 19S class of proteins is a carbohydrate content much larger than that of the low molecular weight group. This consists of hexose and hexosamine sugars

and sialic acid. All the antibodies and other substances of this group that have been tested are very similar in this respect and show approximately 10 per cent by weight carbohydrate.[23,24] The high sialic acid content appears to be responsible at least in part for the more acidic properties of these proteins than are possessed by ordinary low molecular weight antibodies.

Failure to give passive cutaneous anaphylactic reactions. Recent studies by us have indicated that the proteins of this class do not fix to tissues in the same way as do ordinary γ globulin and do not give a passive cutaneous anaphylactic (PCA) reaction on stimulation with the respective antigens (unpublished observations). This has been particularly well documented with

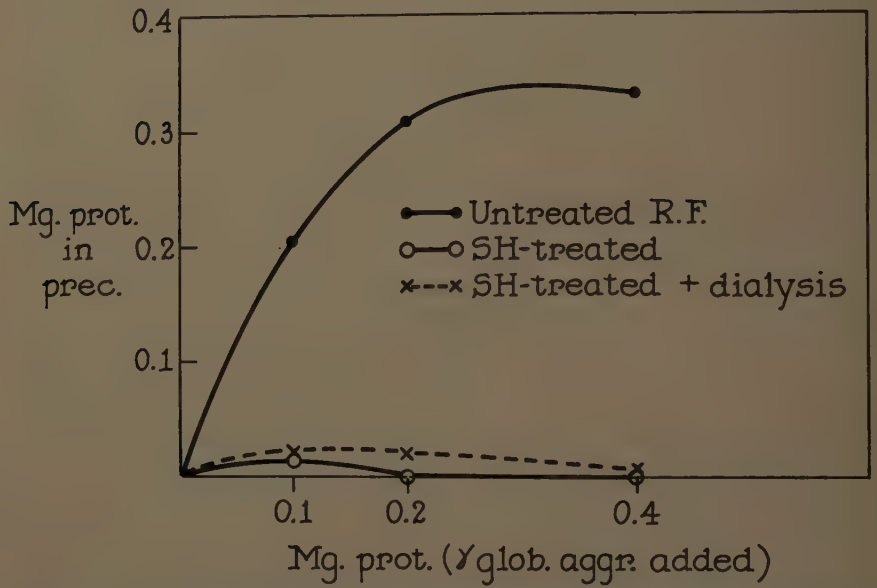

FIGURE 1. Precipitin curves of an isolated preparation of rheumatoid factor (*R.F.*) with increasing amounts of highly purified γ-globulin aggregates. No significant precipitate formed after dialysis against mercaptoethanol and no activity returned after removal of the mercaptoethanol.

respect to the isoagglutinins. It was possible to show that certain anti-β sera give PCA reactions when challenged with B substance and that others do not. Ultracentrifuge analysis indicated that those that failed had the 19S isoagglutinins and those giving the reaction had the 7S type. Antiserum to the 19S proteins failed to give the reaction when the 19S proteins were injected in the skin of guinea pigs, but did react when utilized in the reverse direction and the antiserum was given in the skin.

Dissociation with sulfhydril compounds. One of the most interesting characteristics of this class of materials is the rapid dissociation with SH compounds, due to cleavage of disulfide linkages.[5,24-26] This was first demonstrated for the Waldenström macroglobulins, but now has been shown to apply to a wide variety of antibodies of this type.[5,17,26,27] FIGURE 1 illustrates the effect of treatment with mercaptoethanol on the precipitin curve of rheuma-

toid factor and aggregated γ globulin. All activity disappears and is not regained even when highly purified rheumatoid factor is used and optimal conditions for reassociation are arranged. The serologic tests for rheumatoid factor also are diminished markedly by this procedure. The number of disulfide bonds broken by this procedure is not entirely certain. There appear to be six monomeric units linked by these disulphide bonds, and some evidence suggests that two bonds link each chain.[28]

The sensitivity to mercaptoethanol or cysteine may be used to determine whether or not a given activity belongs in the 19S groups.[17] Prolonged dialysis against the sulfhydril compound has given the best results in our laboratory. It must be realized, however, that a number of factors may influence the results. Different antibodies vary in their ability to split, depending on their concentration and whether they are in whole serum or in the isolated state. If an antibody is readily split by an agent, the likelihood that it is a 19S compound is considerable. However, failure of inactivation indicates considerably less likelihood, and some proved 19S antibodies have been quite resistant under specific conditions.

Detection by Density Gradient Zone Ultracentrifugation

Difficulties have been encountered in determining whether a given antibody belongs in the 7S or 19S class, or both, by the ordinary methods of ultracentrifugation. In certain instances, the presence of antibodies in both classes in the same serum gave very confusing results by preparative ultracentrifugation. Even the use of partition cells in the analytic machine have been of limited value. Considerably greater success has been achieved through the use of a sucrose density gradient, somewhat like that utilized by Brakke for the separation of viruses.[29] The details of this method have been published separately.[30] Serum or protein fraction is layered over the sucrose gradient and then separated in a swinging bucket rotor. Under appropriate conditions the 7S proteins pass approximately one third of the way down the tube, while the 19S proteins go all the way to the bottom. Samples from different levels then may be taken for activity assay. The use of specific antisera to 7S and 19S fractions aids in localizing these classes, although in most instances the 19S material is visible in the tube when a sharp band of light is passed through the bottom in a dark room.

Table 4 shows the results of isoagglutinin assay (anti-A in this case) by this procedure. Three types of patterns were obtained from different sera: pure 19S, pure 7S, and a mixture of 7S and 19S classes. The pure 7S type was the most uncommon, but the mixture was seen with considerable frequency. The older belief that the isoagglutinins are entirely 19S proteins was certainly an oversimplification. The isoagglutinins illustrate the general principle that antibodies are rarely of only the 19S class, and appear also as the ordinary type in individual instances.

Conclusions

The thesis presented earlier, that the word antibody no longer has significant meaning unless the class of antibody is designated, seems valid in view of the many highly specific properties of the 19S class. In the case of the rheumatoid

factors, the high carbohydrate content and the failure to give PCA reactions have been cited as arguments against their antibody nature. It is now known that these are characteristic properties of this class of proteins and apply as well to clearly demonstrated antibodies belonging to this group. A wide variety of antibodylike materials in addition to the rheumatoid factors is being discovered in various connective tissue diseases. The clear definition of these substances as antibodies must await basic studies of well-characterized antibodies belonging to the various subgroups of "immunoglobulins."

References

1. Kunkel, H. G., & R. E. Franzl. Electrophoretic mobility and ultracentrifugal characteristics of pneumococcal antibodies in hyperimmunized rabbits. New England J. Med. In press.
2. Cann, J. R. 1953. Ultracentrifugal properties of human γ-globulins prepared by electrophoresis connection. J. Am. Chem. Soc. **75:** 4213.
3. Wallenius, G., Trautman, R., E. C. Franklin & H. G. Kunkel. 1956. Heavy components of human serum. Federation Proc. **15:** 1.
4. Heremans, J. F., M. T. Heremans & N. E. Schultze. 1959. Isolation and description of a few properties of the β_{2A}-globulin of human serum. Clin. Chim. Acta. **4:** 96.
5. Fudenberg, H. & H. G. Kunkel. 1957. Physical properties of the red cell agglutinins in acquired hemolytic anemia. J. Exptl. Med. **106:** 689.
6. McKay, I. R. 1959. Macroglobulins and macroglobulinemia. Australian Ann. Med. **8:** 158.
7. Kritzman, J. & H. G. Kunkel. A Waldenström type macroglobulin with rheumatoid factor properties. New England J. Med. In press.
8. Burnet, M. 1958. The Clonal Selection Theory of Acquired Immunity. The 1958 Abraham Flexner Lecture. Vanderbilt Univ. Press. Nashville, Tenn.
9. Franklin, E. C., H. R. Holman, H. J. Muller-Eberhard & H. G. Kunkel. 1957. An unusual protein component of high molecular weight in the serum of certain patients with rheumatoid arthritis. J. Exptl. Med. **105:** 425.
10. Edelman, G. M., H. G. Kunkel & E. C. Franklin. 1958. Interaction of the rheumatoid factor with antigen antibody complexes and aggregated γ-globulin. J. Exptl. Med. **108:** 105.
11. Kunkel, H. G., H. H. Fudenberg & H. J. Muller-Eberhard. 1959. Gamma globulin complexes in the sera of certain patients with rheumatoid arthritis (Abstract). Proc. Rheumatic Assoc. Detroit, Mich.
12. Lepow, J. H., L. Pillemer, M. D. Schoenberg, E. W. Todd & R. J. Wedgwood. 1959. The properdin system and immunity. X. Characterization of partially purified human properdin. J. Immunol. **83:** 428.
13. Christenson, W. N., J. V. Dacie, B. E. E. Croucher & P. A. Charlwood. 1957. Electrophoretic studies on sera containing high titer cold hemoagglutinins: identification of the antibody as the cause of the abnormal γ-1 peak. Brit. J. Haematol. **3:** 262.
14. Wiener, A. S., L. J. Unger, L. Cohen & J. Feldman. 1956. Type-specific cold autoantibodies as a cause of acquired hemolytic transfusion reactions: biologic tests with bovine red cells. J. Exptl. Med. **44:** 221.
15. Deicher, H. R. G., H. R. Holman & H. G. Kunkel. 1960. Anti-cytoplasmic factors in the sera of patients with systemic lupus erythematosus and certain other diseases. Arthritis and Rheumatism. **3:** 1.
16. Goodman, H. C., J. L. Fahey, R. A. Malmgren & G. Brecker. 1959. Separation of factors in lupus erythematosus serum reacting with components of all nuceli. Lancet. **2:** 382.
17. Fudenberg, H. H., H. G. Kunkel & E. C. Franklin. 1958. High molecular weight antibodies. : 522. Proc. 7th Congr. Intern. Soc. Blood Transfusion. Rome, Italy.
18. Davis, B. D., D. H. Moore, E. A. Kabat & A. Harris. 1945. Electrophoretic ultracentrifugal and immunochemical studies on Wassermann antibody. J. Immunol. **50:** 1.
19. Muller-Eberhard, H. J., & H. G. Kunkel. 1959. Ultracentrifugal characteristics and carbohydrate content of macromolecular γ-globulins. Clin. Chim. Acta. **4:** 252.
20. Franklin, E. C. & H. G. Kunkel. 1957. Immunologic differences between the 19S and 7S components of normal human γ-globulin. **78:** 11.
21. Korngold, L. & G. Van Leeuwen. 1957. The effect of the antigen's molecular weight on the curvature of the precipitin line in the Ouchterlony technique. J. Immunol. **78:** 172.

22. Burtin, P., L. Hartmann, J. Heremans, J. J. Scheidegger, F. Westendorp-Boerma, R. Wieme, C. Wunderly, R. Fauvert P. & Grabar. 1957. Études Immunochimiques et immuno-électrophoretiques des macroglobulinémies. Rev. Franc. Étud. Clin. et Biol. **2:** 161.
23. Kunkel, H. G., H. J. Muller-Eberhard & E. C. Franklin. 1956. Two types of γ-globulin differing in carbohydrate content. Proc. Soc. Exptl. Biol. Med. **93:** 146.
24. Kunkel, H. G., E. C. Franklin & H. J. Muller-Eberhard. 1959. Studies on the isolation and characterization of the "rheumatoid factor". J. Clin. Invest. **38:** 424.
25. Deutsch, H. F. & J. I. Morton. 1957. Dissociation of human serum macroglobulins. Science. **125:** 600.
26. Grubb, R. & B. Swahn. 1958. Destruction of some agglutinins but not of others by two sulfhydryl compounds. Acta Pathol. Microbiol. Scand. **43:** 305.
27. Deutsch, H. F. & P. C. Y. Chan. 1958. Human serum hemagglutinins. Federation Proc. **17:** 210.
28. Edelman, G. M. 1960. Structural studies of antibody globulins. Doctoral Dissertation. Rockefeller Inst. Press. New York, N. Y.
29. Brakke, M. K. 1953. Zonal separation by density gradient centrifugation. Arch. Biochem. Biophys. **45:** 275.
30. Kunkel, H. G. 1960. Macroglobulins and high molecular weight antibodies. *In* Plasma Proteins. F. W. Putnam, Ed. Academic Press. New York, N. Y.

THE CONCEPT OF AUTOANTIBODIES IN RHEUMATIC FEVER AND IN THE POSTCOMMISSUROTOMY STATE*

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It is generally accepted that infection of the upper respiratory tract by the group A streptococcus is the essential event provoking a primary attack of acute rheumatic fever. Following the primary attack, two eventualities may be considered: (1) the disease may progress chronically or cyclically with permanent damage to myocardium and heart valves, with no detectable evidence of concomitant foci of streptococcal infection (although this point recently has been challenged[1,2]); and (2) there may be a complete recovery with or without residuum, followed, however, by a marked susceptibility to recurrent attacks of rheumatic fever.[3,4]

Over the years numerous hypotheses have been proposed in attempts to dovetail the biological properties of the group A streptococcus with the special peculiarities of host behavior and tissue response that characterize this disease. None of these has been entirely satisfactory, as is suggested, for example, by the variety of experimental or clinical studies published within recent years dealing with the possible mechanisms of cardiac damage. These have concerned:

(1) The pathological effects on the heart of streptococcal products, including enzymic substances, studied by Kellner and Robertson,[5] Carlson *et al.*,[6] Robinson,[7] and Wagner (elsewhere in this monograph).

(2) Cardiac lesions, especially of myofibers, resulting from repeated focal streptococcal infections of the rabbit skin, and the morphologic resemblance of such lesions to those observed in rheumatic carditis, as described by Murphy and Swift,[8] Murphy,[9] and Kirschner and Howie.[10]

(3) Cardiac lesions following establishment of focal infection of subcutaneous or pharyngeal tissue in the rabbit, studied by Glaser and his associates.[11]

(4) The demonstration of the localization of streptococcal antigen in the heart, as a possible focus for allergic reaction, by Kaplan.[12]

(5) The clinical and epidemiological studies of Rammelkamp and his associates[1] indicating necessity of *persistence* of viable streptococci in the tissues for activation of rheumatic fever.

(6) The demonstration of leukocyte-platelet thrombi in rheumatic hearts and studies of the possible relationship of this finding to the Shwartzman phenomenon or to the Arthus phenomenon type of hypersensitivity, by Stetson.[13]

(7) Finally, the participation of an autoimmune mechanism in pathogenesis, first suggested by the clinical studies of Brockmann *et al.*[14] in Germany in 1937 and given impetus by the work of Cavelti[15] in the United States in 1945.

It is the purpose of this paper to review critically this last proposition, the

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autoimmune concept of rheumatic fever, and to summarize some recent observations made in our own laboratory and to be published in detail elsewhere.

The hypothesis that an immunological mechanism in some form is involved in the pathogenesis of rheumatic fever is probably supported by the majority of the students of this disease. The arguments pertaining to this question recently have been reviewed authoritatively and critically by McCarty.[16] However, the published evidence that this mechanism may be one of autoimmunity has not been convincing, either from immunological or clinical points of view.

First, from the point of view of experimental immunology, the heart has not been established as a source of organ-specific antigen with potential for the production of autoantibodies as have the brain, thyroid, lens, or testis. Cavelti[17,18] in 1947 first presented experimental evidence favoring heart tissue as a source of autoimmunizing antigen. He reported that repeated intraperitoneal injections of rats with a mixture of homogenized rat heart tissue and killed group A streptococci over a period of 1 to 3 months resulted in the production in the serum of agglutinins for collodion particles coated with a saline extract of heart tissue in 45 to 80 per cent of animals so treated. In approximately 30 to 50 per cent of the animals, lesions involving endocardium, myocardium, and epicardium were produced. They were related particularly to *connective tissue* elements and were notable in valves, atrioventricular ring, blood vessels, and the interstitial tissue of the myocardium. An important point was that *myocardial fibers were spared*, unless involved by contiguous structures. *Confirmation of these results has never been reported.*

In the same year (1947) Jaffe and Holz[19] independently described myocardial lesions in rats and rabbits immunized for 9 months with a mixture of homologous heart tissue and killed streptococci. The pathological description of the lesions and the published illustrations do not correspond with the lesions described by Cavelti. In their illustrations the lesions appeared to involve the myocardium chiefly and the connective tissue to a lesser degree, and were characterized by the authors as consisting primarily of muscle degeneration with secondary inflammation. No serologic studies, however, were carried out to prove an isoallergic basis for these lesions.

The Japanese investigators Maekawa[20] and Shoji[21] have presented work suggesting that a cephalin fraction of mammalian heart tissue injected in admixture with foreign serum or other protein serving as *carrier* may induce interstitial myocarditis. The lipoid fraction was regarded as organ-specific. The brief descriptions and illustrations are chiefly of an interstitial myocarditis, although myofiber degeneration and vascular lesions were also noted. No serologic studies were carried out.

In a German publication in 1951, Frick[22] reported lesions in rats following repeated intraperitoneal injections of homologous heart in Freund's adjuvant plus tuberculin. These were not described in detail, but revealed connective tissue damage in perivascular distribution, myocardial hemorrhages and, in a minority of animals, focal areas of myocardial degeneration. Sera were examined for autoantibodies by complement fixation, with a suspension of rat heart tissue as antigen. However, only 3 of the 11 animals in which lesions were produced exhibited such antibodies.

More recently in our laboratory[23,24] it has been possible to demonstrate production of antibodies reactive with autologous heart in rabbits immunized with heterologous heart tissue homogenates such as beef or rat, as revealed by immunofluorescence and complement fixation. The antigenic material was identified as a cross-reacting lipoid constituent of mammalian cardiac muscle. Injection of rabbits with fresh homologous heart tissue and Freund's adjuvant has failed to yield such antibodies.[25]

In sum, these reports still place the experimental production of autoantibodies to heart on tenuous grounds. The role of such autoantibodies in the production of cardiac lesions must be considered unconfirmed.

In the clinical area of investigation, several workers have reported the presence of presumptive autoantibodies to heart in sera of patients with rheumatic fever, as revealed by complement fixation, collodion particle agglutination, and antiglobulin consumption tests. The specificity of these tests from both clinical and immunologic points of view has been disputed. Brockmann *et al.*[14] reported in 1937 a high incidence of complement-fixing antibodies to saline extracts of heart and liver tissue derived from a rheumatic patient in the sera of children with acute rheumatic fever, in adults with acute or chronic polyarthritis and, only occasionally, in normal persons or patients with other types of inflammatory disease. Extracts of rheumatic heart and spleen were less effective than was liver as antigen, but extracts of nonrheumatic tissues were said to be entirely ineffective.

Cavelti[15] in 1945 used collodion particles coated with saline extracts from normal (nonrheumatic) heart as antigen, and found that the extracts from 1 of 4 hearts tested gave positive agglutination reactions in the sera of 27 of 36 patients with acute rheumatic fever, in none of 12 normal sera, and in only 1 of 84 sera of nonrheumatic patients with various diseases. It was Cavelti's impression that these agglutinins were present in the "early and most active stages and disappeared when the rheumatic process became inactive." Cavelti is quoted later by Fischel and Pauli[26] as having had difficulty in repeating these results with tissue extracts other than from the one heart that gave strongly positive results. These latter workers failed to confirm the work of Brockmann *et al.*[14] and, in attempts to repeat the work of Cavelti,[15] noted only infrequent reactions with heart and other organ extracts by collodion particle agglutination. It was suggested that such tests were not specific for rheumatic fever, and that the results were comparable to "biological false positive" flocculation reactions.

This collodion particle agglutination technique was used also by the Czechoslovakian workers Rejholec and Wagner[27] in 1955, who observed positive reactions in the sera of 6 of 8 patients with rheumatic fever, and also in the sera of a small number of patients with acute tonsillitis, rheumatoid arthritis, and glomerulonephritis, as well as occasionally in the sera of a mixed control group consisting of normal persons and patients with various diseases. These investigators stated that in rheumatic fever these antibodies receded or disappeared with salicylate treatment.

In 1954, in Baltimore, Osler *et al.*[28] noted that the sera of nonsyphilitic persons frequently contained complement-fixing antibodies to alcoholic extracts of hu-

man heart tissue. A large proportion of these extracts came from patients with rheumatic fever. Thus, of 259 sera of cases of rheumatic fever or rheumatic heart disease, 117, or 45 per cent, were definitely positive while, of a miscellaneous control group of 254 sera, 54, or 21 per cent, were definitely positive. Positively reacting sera showed no fixation when tested with beef heart cardiolipin. In patients with rheumatic disease, no obvious correlation was noted between the serum titer and the course of the disease process. In addition, as compared with a 20 per cent incidence of positive tests in normal healthy individuals, there was noted a distinct increase in the frequency of such reactions in patients with parenchymatous disease involving liver, brain, thyroid, kidney, and also with blood dyscrasias.

Most recently, two reports in the German literature, by Butler and Moeschlin[29] and by Steffen,[30] describe presumptive autoantibodies to heart tissue shown by the antiglobulin consumption technique, using lyophilized normal heart tissue as antigen. The sera from a small number of patients with rheumatic fever and rheumatoid arthritis were found positive.

Thus, from these reports in the clinical area, it appears that patients with rheumatic fever frequently may exhibit presumptive autoantibodies to heart tissue; however, controversy exists as to the clinical specificity of the serologic tests employed. In all these reports, except that of Cavelti, positive serologic responses were observed in diseases other than rheumatic fever. These divergent results probably cannot be explained in face of the unknown nature of the antigen or antigens employed.

The interest of our laboratory in the autoimmunity concept stemmed from the results of a survey of auricular biopsy material obtained from patients with rheumatic heart disease who had come to cardiac surgery. In this study, begun in 1954, auricular tissues were examined for the presence of bound gamma globulin by using the immunofluorescent technique, on the *hypothetical* assumption that the presence of such bound gamma globulin was consistent with, although not proof of, immunological mechanism in pathogenesis.

One hundred auricular appendages were made available for study in Boston and Cleveland from 1954 to 1959.* Of these 100 specimens, 18 gave unequivocal evidence of bound gamma globulin† with characteristic histological distribution. Moreover, 2 of 4 specimens of hearts from patients dying of acute rheumatic fever exhibited bound gamma globulin. These deposits were not associated with Aschoff lesions; they occurred in spotty distribution throughout the myocardium, involving especially myocardial cells including sarcolemma and subsarcolemmal sarcoplasm and, in addition, interstitial connective tissue and vessel walls. For control observations, 30 specimens were available from nonrheumatic hearts, including 28 post-mortem and 2 auricular biopsy specimens. These exhibited focal myocardial fibrosis in 9 cases, acute myocardial infarction in 4, pericarditis in 2, interstitial fibrosis in 1, and myxoma in 1, the

* We are grateful to Dwight E. Harken, Gustave Dammin, Gordon Scannell, and Lawrence Kunz, of Boston, Mass., and George H. Clowes, of Cleveland, Ohio, for making these tissues available.

† It should be stressed that by *bound* gamma globulin is meant gamma globulin that cannot be removed from the tissues following repeated washings of unfixed frozen sections, in contrast to the completely diffusible and extractable gamma globulin of normal or pathological control heart tissue.

remainder being normal.* Of these control tissues, none showed significant deposits of bound gamma globulin; the auricular biopsy tissue from the patient with myxoma showed some traces only in the interstitial connective tissue.

The histological distribution of bound gamma globulin is shown in FIGURES 1 and 2. Scattered focal deposits were noted most frequently within myocardial cells extending from peripheral segments of sarcolemma into the subsarcolemmal sarcoplasm or into the substance of the sarcoplasm. Within the sarcoplasm the gamma globulin occurred as amorphous masses, rods, or membranous deposits between myofibrils. Such gamma globulin-containing cells were distributed irregularly throughout the myocardium of both auricle and ventricle. The interstitial connective tissue also revealed frequent scattered deposits of material, often adjacent to involved myofibers. Rarely, amorphous deposits or droplets were noted within collagenous septa. Finally, bound gamma globulin was noted within segments of vessel walls, particularly of arterioles and venules.

Deposition of gamma globulin was apparently selective, since other proteins, that is, albumin and fibrinogen, were absent from involved foci. Of particular interest was the marked affinity for eosin shown by tissue sites containing bound gamma globulin. This affinity could be recognized in many instances in parallel sections stained by routine hematoxylin-eosin. It was also effectively demonstrated by fluorescence microscopy, with highly dilute solutions of eosin as a fluorochrome stain (FIGURE 3). Tissue sites with affinity for eosin emitted brilliant yellow fluorescence, while normal sections were not stained at the test dilution. The sites of affinity for eosin included all the foci of gamma globulin distribution described above, including myofibers, connective tissue, and vessel walls. The finding of intensely eosinophilic-staining myofibers comparable in intensity to fibrinoid in rheumatic hearts has also been described recently by Murphy.[31] A further evidence of tissue alteration at the sites of gamma globulin deposition was the observation that such sites gave intense staining with the Schiff periodic reagent.

Thus a selective deposition of gamma globulin was associated with objective evidence of tissue alteration in a significant proportion of the rheumatic heart specimens. Could this deposition of gamma globulin be related to an autoimmune reaction with one or more constituents of heart tissue, resulting in tissue damage? It was in an attempt to answer this question that the sera of patients with rheumatic fever and rheumatic heart disease were examined for possible reactivity with normal or rheumatic heart tissue sections. The method of detecting such reactivity was the indirect immunofluorescence technique, and the agent employed for this purpose was fluorescein-labeled rabbit antihuman gamma globulin.

The results of such tests indicated that serologic reactivity with heart tissue could indeed be detected in the sera of certain patients with rheumatic fever or rheumatic heart disease. Two serologic factors, in fact, were distinguished. The first of these yielded a staining pattern with myocardium that involved especially the outward edge of the subsarcolemmal sarcoplasm of the myofiber,

* The collaboration of Frederick Dallenbach, of the Department of Pathology, Peter Bent Brigham Hospital, Boston, Mass., in reviewing many of the histological sections of both rheumatic and nonrheumatic hearts is gratefully acknowledged.

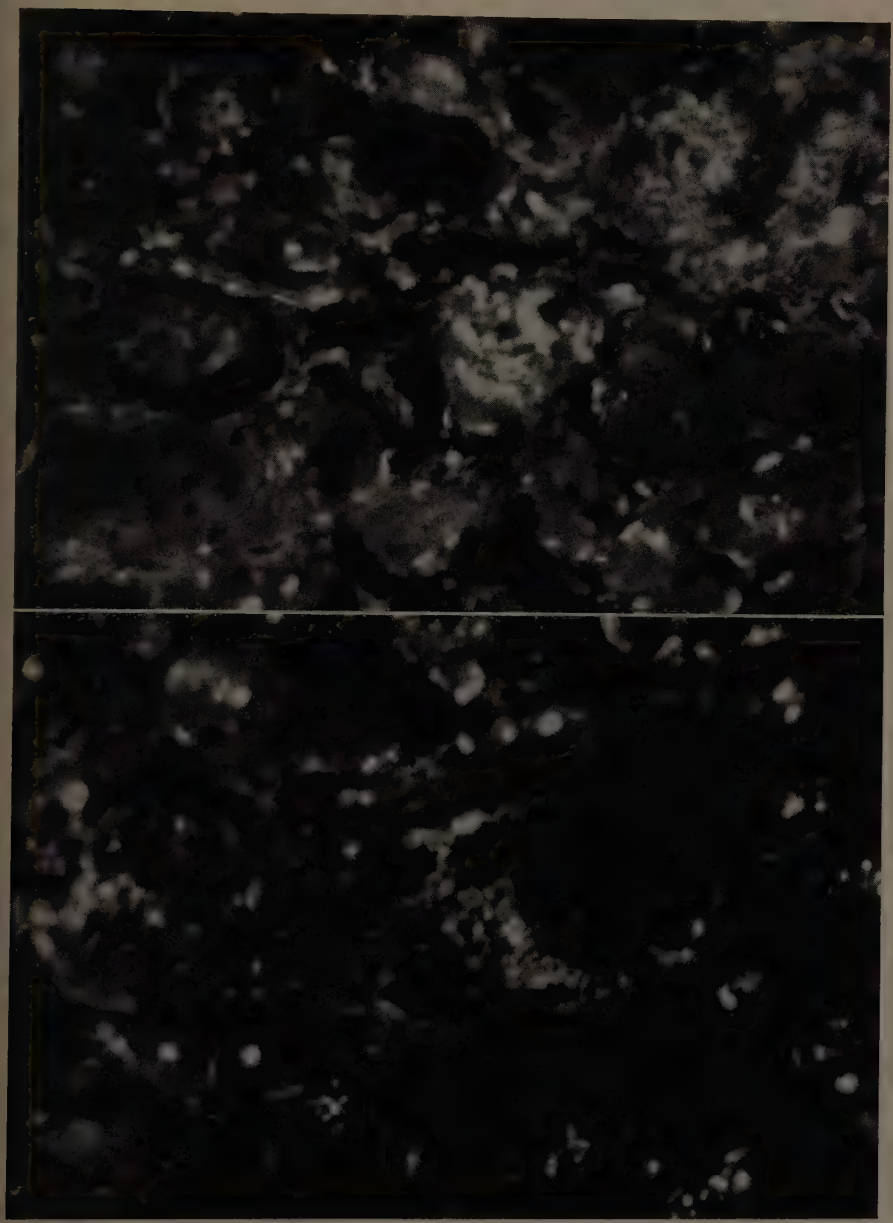

FIGURE. 1. *Top*, auricular appendage (patient For); bound gamma globulin within myocardial cells, as deposits within substance of sarcoplasm as well as in sites between myofibrils and in sarcolemma; lesser deposits in interstitial connective tissue. ×400. *Bottom*, auricular appendage (patient For); bound gamma globulin within myocardial cell, in spaces between myofibrils as well as in interstitial connective tissue and sarcolemma of neighboring cells; large round or ovoid bodies contain red fluorescent pigments. ×400. Lightest areas are green fluorescence due to immunofluorescent reaction, in this and following photographs.

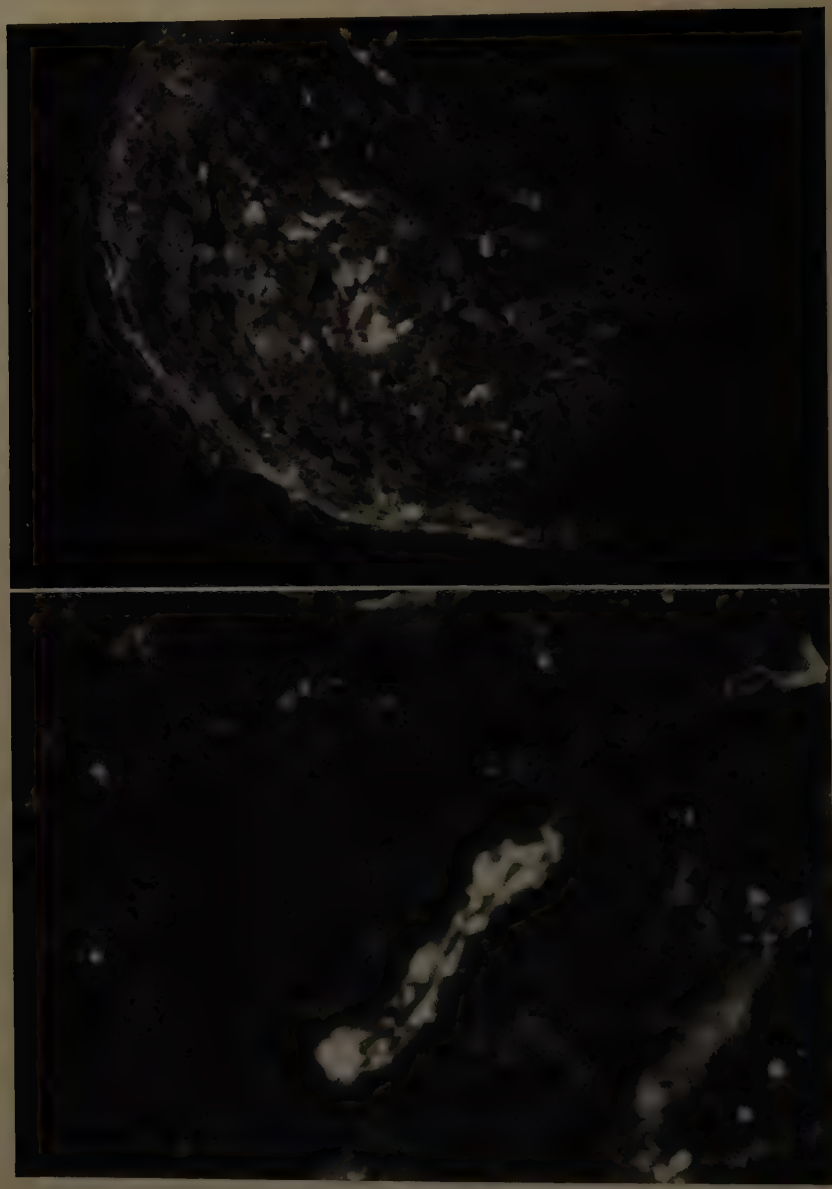

FIGURE 2. *Top*, auricular appendage (patient Lau); low-power view of section exhibiting bound gamma globulin in focal scattered deposits within myocardial cells, interstitial connective tissue, and vessel walls. ×100. *Bottom*, auricular appendage (patient For); bound gamma globulin in wall of arteriole and in sarcolemma of neighboring myofibers, as well as in interstitial connective tissue. ×400.

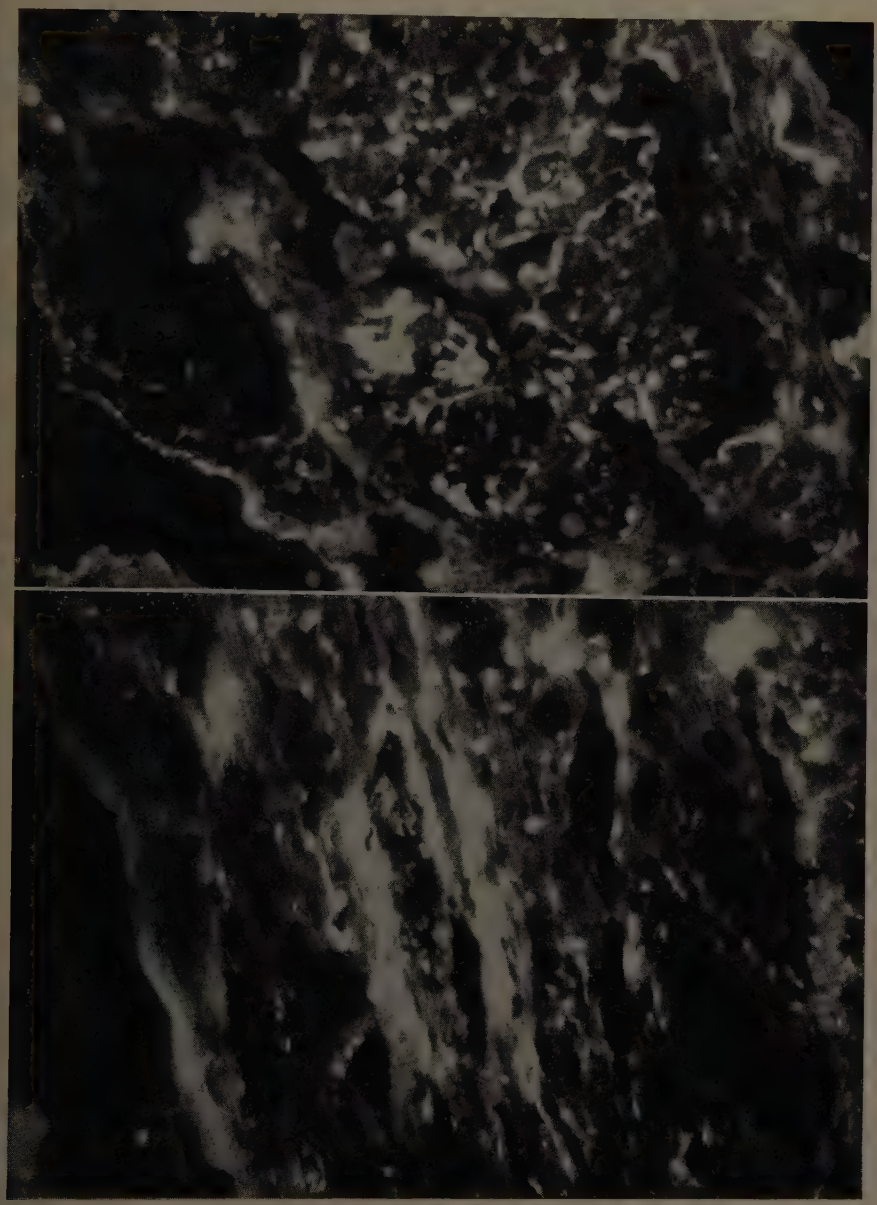

FIGURE. 3. *Top*, auricular appendage (patient For); section is stained with dilute eosin as fluorochrome; sites of eosin binding include amorphous and rodlike masses within myofibers, sarcolemma, interstitial connective tissue, and vessel walls; normal or pathological control heart sections are nonreactive at this eosin concentration; compare with bound gamma globulin distribution in FIGURE 1 (*top*). ×200. *Bottom*, auricular appendage (patient Lau); section stained with dilute eosin as fluorochrome; segments of myofibers, including sarcoplasm and sarcolemma, show marked affinity for eosin. ×200.

usually with a lesser diffuse reaction with the substance of the sarcoplasm. This subsarcolemmal-sarcoplasmic pattern is illustrated in FIGURE 4. The second reactive pattern involved sites within the sarcoplasm in juxtaposition to the myofibrils. This pattern, given the name *intermyofibrillar*, is illustrated in FIGURE 5. Both types of histological pattern were observed in sera reactive with autologous as well as with homologous heart, and with nonrheumatic as well as with rheumatic hearts. Serologic reaction due to blood group isoantibodies involved capillaries and connective tissue, but not myofibers, as shown in FIGURE 6.

For confirmation of these two immunofluorescent staining reactions more traditional serologic methods were used. Inasmuch as both patterns of reaction could be abolished completely by treatment of the sections with ethanol or methanol and not with acetone or ether, it was assumed that the antigens were alcohol-extractable and that serologic tests might be developed in which alcoholic extracts of human heart were used as antigen. It was recalled that Osler *et al.*[28] had successfully demonstrated, some years ago, positive complement fixation reactions with alcoholic extract of human heart in the sera of a large proportion of patients with rheumatic fever and of a lesser proportion of patients with certain other conditions. Alcoholic extracts of human heart were prepared according to the methods of these authors, and complement fixation tests were carried out with positive sera that had exhibited either subsarcolemmal or intermyofibrillar patterns of reactivity. The serologic properties of the two types of sera were different. Complement fixation was obtained with subsarcolemmal-reactive sera, but not with intermyofibrillar-reactive sera. With respect to the former, a fair correlation could be demonstrated between the complement fixation and immunofluorescent tests. The intermyofibrillar-reactive sera, however, as well as subsarcolemmal-reactive sera gave positive flocculation reactions with alcoholic extracts of heart. Demonstration of the flocculation reaction was best carried out with the presence of polystyrene latex particles treated with a fresh alcoholic extract of human heart. The results are shown in TABLE I. Correlation between latex tests and immunofluorescence was not perfect, since occasional sera were encountered with positive flocculation and negative fluorescence, as noted with the last serum listed in the table. The specificity of the immunofluorescent staining, however, was supported by absorption tests with cholestrinized alcoholic extracts of human heart, which completely abolished the staining reactions of both subsarcolemmal and intermyofibrillar patterns.

With fluorescent antibody specific for 19S gamma globulin,[32] the serologic activity of the 19S component of sera could be tested by the *indirect* immunofluorescent method. Of 50 sera reacting positively with heart tissue, as determined with fluorescent anti-7S gamma globulin, only 4 were reactive also when fluorescent anti-19S gamma globulin was employed. The pattern was of the subsarcolemmal type in these 4 cases. Only 1 patient has been observed with reactivity due to 19S gamma globulin alone.

The frequency of occurrence of these 2 serologic factors in the sera of 500 patients selected from a general hospital population is shown in TABLE 2. Of this group, 16.8 per cent gave positive reactions. Most of these showed the

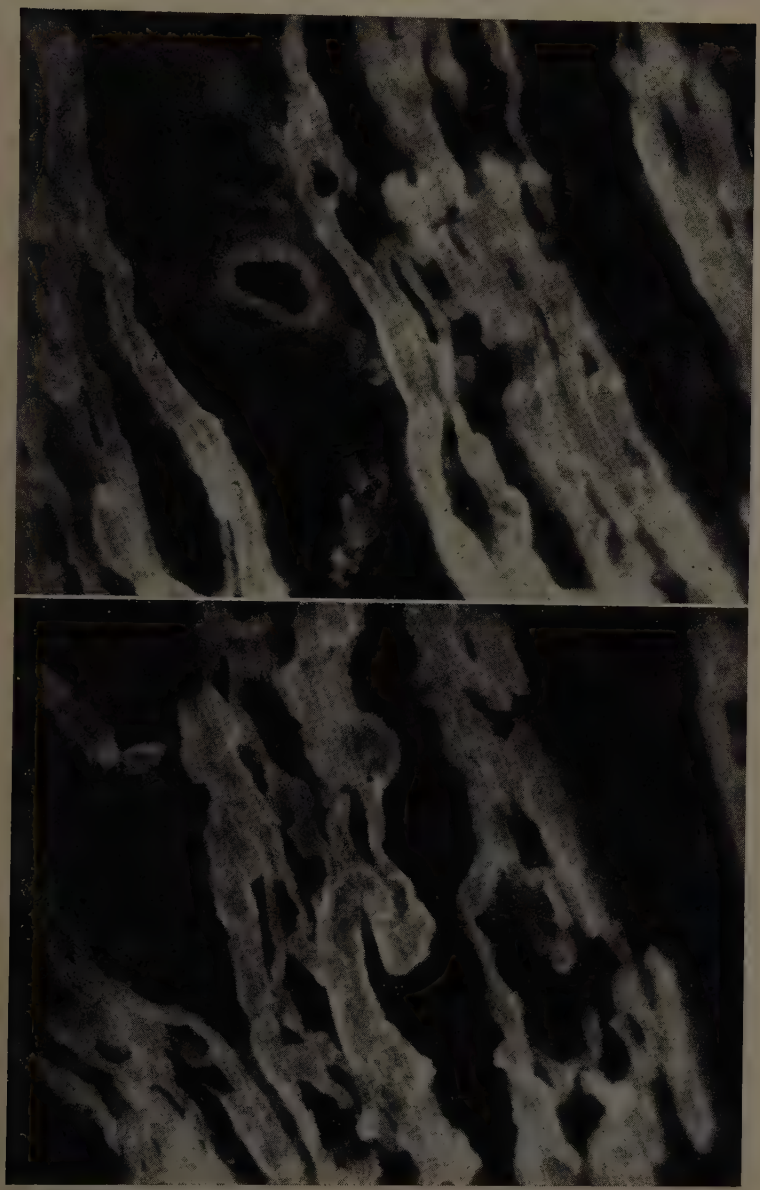

FIGURE. 4. *Top*, subsarcolemmal-sarcoplasmic pattern of staining exhibited by interaction of serum from patient with rheumatic carditis and normal heart; vessel wall and adventitial and interstitial connective tissue nonreactive. ×200. *Bottom*, subsarcolemmal-sarcoplasmic pattern of staining given by serum from postcommissurotomy patient reactive with normal heart. ×200.

subsarcolemmal pattern. In TABLE 3 the clinical categories of the positively reacting sera are given. Neither of the 2 reactive patterns was found specific for rheumatic fever or rheumatic heart disease; positive reactions were detected also in certain patients with rheumatoid arthritis, disseminated lupus, chronic

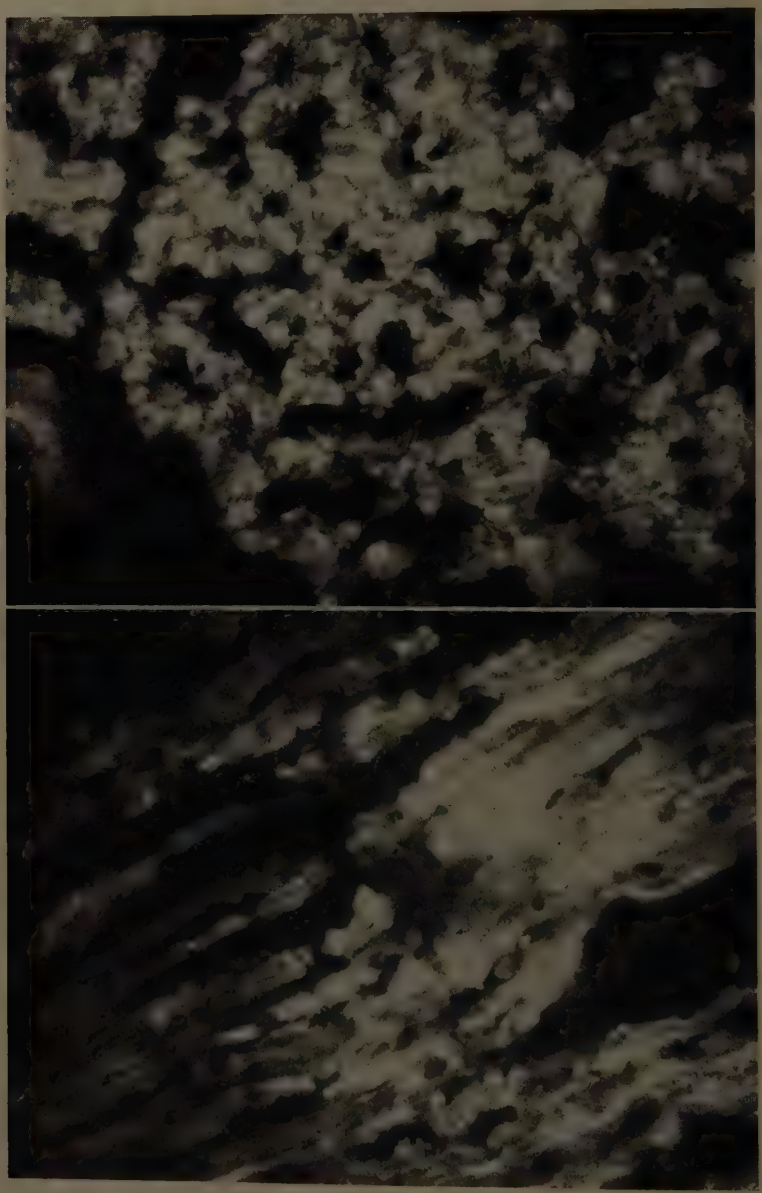

FIGURE 5. *Top*, intermyofibrillar pattern of staining exhibited by interaction of post-commissurotomy serum and normal heart. ×200. *Bottom*, intermyofibrillar pattern; post-commissurotomy serum applied to normal heart; longitudinal view. ×200.

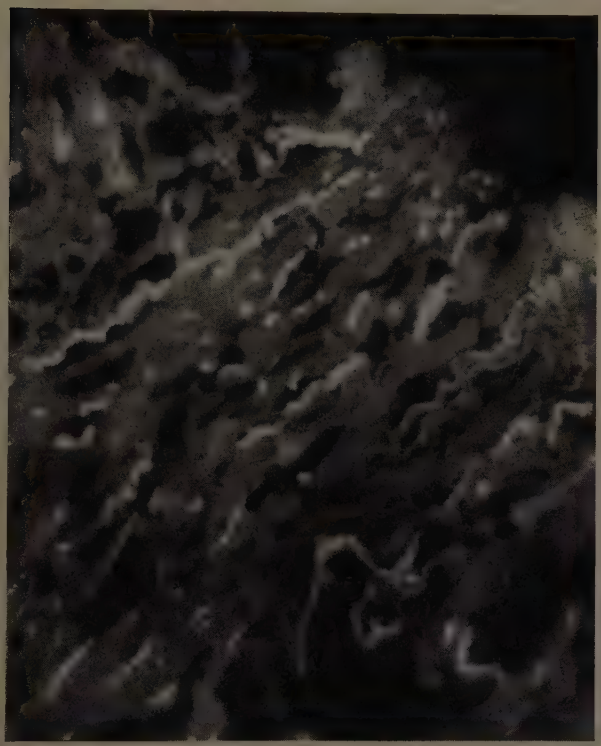

FIGURE 6. Pattern given by isoimmune bodies to blood group substance A, present in this heart tissue within the capillary walls and interstitial connective tissue; no reaction is observed with myofibers, indicating that heart-reactive factors are absent in this serum. ×200.

TABLE 1

SEROLOGIC REACTION WITH ALCOHOLIC EXTRACT OF HUMAN HEART BY LATEX PARTICLE AGGLUTINATION TECHNIQUE

Serum no.	Serum titer	Immunofluorescent staining pattern
1010	8	Ssl*
1400	32	Ssl
1350	32	Ssl
2691	32	Ssl
1176	32	Imf†
2724	32	Imf
2403	16	Imf
1754	0	0
2028	0	0
1240	0	0
2582	16	0

* Subsarcolemmal pattern of serum reaction.
† Intermyofibrillar pattern of serum reaction.

liver disease, and in certain other conditions. Of special significance was the observation that positive responses were encountered most frequently in patients with rheumatic heart disease, following cardiac surgery. Furthermore,

TABLE 2

FREQUENCY OF OCCURRENCE OF SUBSARCOLEMMAL AND INTERMYOFIBRILLAR REACTIVE FACTORS IN THE SERA OF 500 HOSPITALIZED PATIENTS, AS DETERMINED BY THE IMMUNOFLUORESCENT METHOD

No. of sera tested	500
No. of sera positive	84 (16.8%)
No. of sera giving positive reaction of subsarcolemmal pattern	67 (13.4%)
No. of sera giving positive reaction of intermyofibrillar pattern	17 (3.4%)

TABLE 3

DISTRIBUTION OF POSITIVELY REACTING SERA BY CLINICAL CATEGORY

Diagnostic category	No. of sera within category	No. reactive with subsarcolemma	No. reactive with intermyofibrillar sites
Acute rheumatic fever	40	10 (25%)	0 (0%)
Rheumatic heart disease	67	10 (14.9%)	3 (4.5%)
Rheumatic heart disease postvalvuloplasty	39	27 (69%)	6 (15.4%)
Rheumatoid arthritis	27	5 (18.5%)	1 (3.7%)
Systemic L.E.	11	2 (18.2%)	3 (27.2%)
Chronic liver disease	38	7 (18.4%)	2 (5.3%)
Miscellaneous*	8	6	2

* Subsarcolemmal reactions were noted with sera from one each of the following cases: anaphylactoid purpura, serum sickness, paroxysmal nocturnal hemoglobinuria, idiopathic pulmonary hemosiderosis, lung carcinoma, and acute streptococcal pharyngitis.

Intermyofibrillar reactions were noted with sera from one each of the following cases: anaphylactoid purpura and scleroderma.

TABLE 4

SEROLOGIC REACTIONS OF RHEUMATIC PATIENTS WITH AUTOLOGOUS HEART PRE- AND POST-CARDIAC SURGERY AS DETERMINED BY IMMUNOFLUORESCENT REACTION*

Patient no.	Subsarcolemmal reaction 0 to 5 days before surgery	Subsarcolemmal reaction 8 to 21 days after surgery
1	0	++++
2	0	++++
3	0	+++
4	0	+++
5	0	+++
6	0	+++
7	0	++

* Intensity of staining reaction with autologous heart graded from 0 to ++++.

as shown in TABLE 4, this serologic activity appeared in the sera in 1 to 3 weeks following cardiac surgery, suggesting that surgical damage to the heart serves as a stimulus for eliciting these factors reactive with autologous tissue. From 1 patient with postcommissurotomy syndrome, auricular tissue, pre- and post-valvuloplasty sera, and pleural fluid specimens were available for study, and

revealed an enhanced reactivity to autologous heart in postvalvuloplasty sera and pleural fluid.

Since, as described above, bound gamma globulin could be detected within the cardiac myofibers in a significant proportion of patients with rheumatic heart disease who come to cardiac surgery, is it possible to conclude that the gamma globulin in the auricular biopsy specimens is derived from circulating autoantibodies to myofiber constituents that have become fixed *in vivo*? This seems to be the simplest and most logical interpretation of the data. However, it may be too simple.

First, the presence of heart-reactive factors was detected in sera of cases in which cardiac involvement was not evident. Questions are thus raised: first, as to the pathogenetic significance of these "autoantibodies" and, second, as to the organ-specificity of the autoimmunizing antigens. The question of organ specificity has not yet been fully investigated, although preliminary immunofluorescent experiments indicate that the reactive antigens are present also in the striated muscle of the skeletal system.

Next, bound gamma globulin in the rheumatic hearts was noted also in interstitial connective tissue and in vascular walls. It is conceivable that gamma globulin deposited in the interstitium represents either a reaction of autoantibodies with sarcoplasmic antigens released from myocardial cells into these sites or a distribution of the same materials as antigen-antibody complexes. Localization of antigen-antibody complexes in vessel walls in experimental serum sickness has been described by Dixon and his associates.[33] On the other hand, other possibilities must also be considered, for example, association of the gamma globulin in connective tissue and vascular walls with separate immunological systems related to these sites or, alternatively, association of bound gamma globulin with an unknown nonimmunological mechanism of tissue alteration or inflammation. These considerations relating to the nature and function of gamma globulin deposits summarize the difficulties of interpretation inherent in all recent studies of the lesions of connective tissue in which fluorescent antibody methods were used (see Kaplan[34] for a review of this problem). The theoretical possibilities stated here are neither supported nor negated by present observations.

With respect to the possible relationship of the group A streptococcus to the observations reported in this paper, no satisfactory data can be presented. Only 25 per cent of our patients with acute rheumatic fever* who exhibited carditis on admission showed the presence of serum factors. Clinical analysis of this group will be presented in detail subsequently. The hypothesis that one or more toxic substances elaborated by the streptococcus will initiate the cardiac or tissue damage responsible for stimulation of these serum factors is conceivable, but also remains to be proved.

On the other hand, the high frequency of occurrence of heart-reactive factors in the sera of rheumatic patients following cardiac surgery suggests strongly that the damage to heart tissue during cardiotomy is the factor evoking production of these autoimmune bodies. It is as yet not clear how the presence of serum factors reactive with muscle can be reconciled with the clinical signs and

* The collaboration of Benedict F. Massell in the selection of patients included in this study is acknowledged with pleasure.

symptoms of the postcommissurotomy syndrome, which usually affects pleura and pericardium and only infrequently myocardium.[35-37] Similar consideration perhaps might be given to the postinfarction syndrome[38] and other postcardiotomy and postpericardiectomy syndromes[39] as analogous clinical manifestations following reaction to damaged myocardium. Whether these serum factors in themselves have pathogenetic significance or whether they reflect the participation of a separate immunological mechanism of more direct importance, within a population of altered or increased immunologic responsiveness, seem to be the central problems next to be explored.

Summary

Previous reports of presumptive autoantibodies to heart tissue in the sera of patients with rheumatic fever are reviewed, as well as the immunological literature relating to heart tissue as a source of autoantibody.

In studies of rheumatic hearts, bound gamma globulin was detected in 18 of 100 auricular appendages and in 2 of 4 hearts of patients dying of acute rheumatic carditis. These deposits occurred throughout the myocardium, involving especially segments of sarcolemma, subsarcolemmal sarcoplasm, interstitial connective tissue, and vessel walls. Normal or pathological control heart tissues were negative. Associated with these gamma globulin-containing sites were evidences of tissue alteration, as indicated by enhanced affinity for eosin, and intense reactivity with the periodic acid–Schiff stain.

Sera from certain patients with rheumatic fever, rheumatic heart disease, and a few other conditions demonstrated marked reactivity with homologous or autologous heart tissue, as revealed by immunofluorescence, complement fixation, and agglutination tests. At least 2 separate alcohol-soluble constituents of myocardial cell sarcoplasm were recognized as reactants. Serologic activity in most sera was attributable to gamma globulins of the 7S species; in a few sera, this reactivity was due also to 19S gamma globulin. These reactive factors occurred with particularly high frequency (85 per cent) in the sera of patients following mitral valvuloplasty.

The finding of bound gamma globulin in myocardial cells of rheumatic tissues in a pattern similar to the immunofluorescent reactivity of these autoantibodies raises the question of their participation in the pathogenesis of rheumatic heart disease. The participation of an immunological mechanism in the pathogenesis of the postcommissurotomy syndrome is suggested. The requirement of evidence in stringent proof of these hypotheses is discussed.

Acknowledgment

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References

1. Catanzaro, F., C. A. Stetson, A. J. Morris, R. Chamovitz, C. H. Rammelkamp, Jr., B. L. Stolzer & W. D. Perry. 1954. The role of the streptococcus in the pathogenesis of rheumatic fever. Am. J. Med. **17**: 749.

2. Mortimer, E. A., S. Vaisman, I. Vignau, J. Guasch, A. Schuster, L. Rakita, R. M. Krause, R. Roberts & C. H. Rammelkamp, Jr. 1959. The effect of penicillin on acute rheumatic fever and valvular heart disease. New Engl. J. Med. **260:** 101.
3. Bland, E. F. & T. D. Jones. 1951. Rheumatic fever and rheumatic heart disease. A twenty-year report on 1,000 patients followed since childhood. Circulation. **4:** 836.
4. Mortimer, E. A., Jr. & C. H. Rammelkamp, Jr. 1956. Prophylaxis of rheumatic fever. Circulation. **14:** 1144.
5. Kellner, A. & T. Robertson. 1954. Myocardial necrosis produced in animals by means of crystalline streptococcal proteinase. J. Exptl. Med. **99:** 495.
6. Carlson, A. S., A. Kellner & A. W. Bernheimer. 1956. Selective inhibition by preparations of streptococcal filtrates of the oxidative metabolism of mitochondria procured from rabbit myocardium. J. Exptl. Med. **104:** 577.
7. Robinson, J. J. 1951. Attempts to produce rheumatic carditis in laboratory animals by means of streptococcic injury. A.M.A. Arch. Pathol. **51:** 602.
8. Murphy, G. E. & H. F. Swift. 1950. The induction of rheumatic-like cardiac lesions in rabbits by repeated focal infections with group A streptococci. J. Exptl. Med. **91:** 485.
9. Murphy, G. E. 1952. Evidence that Aschoff bodies of rheumatic myocarditis develop from injured myofibers. J. Exptl. Med. **95:** 319.
10. Kirschner, L. & J. B. Howie. 1952. Rheumatic-like lesions in the heart of the rabbit , experimentally induced by repeated inoculation with hemolytic streptococci. J. Pathol. Bacteriol. **64:** 367.
11. Glaser, R. J., W. A. Thomas, S. I. Morse & J. E. Darnell, Jr. 1956. The incidence and pathogenesis of myocarditis in rabbits after group A streptococcal pharyngeal infections. J. Exptl. Med. **103:** 173.
12. Kaplan, M. H. 1958. Localization of streptococcal antigens in tissues. I. Histologic distribution and persistence of M protein, types 1, 5, 12, and 19 in the tissues of the mouse. J. Exptl. Med. **107:** 341.
13. Stetson, C. 1957. Occurrence of leukocyte-platelet thrombosis in rheumatic carditis. J. Exptl. Med. **94:** 493.
14. Brockmann, H., J. Brill & J. Frendzell. 1937. Komplement ablenkung mit organ extrakten von rheumatiken bei sogenannten gelenk rheumatismus. Klin. Wochschr. **16:** 502.
15. Cavelti, P. A. 1945. Autoantibodies in rheumatic fever. Proc. Soc. Exptl. Biol. Med. **60:** 379.
16. McCarty, M. 1956. Nature of rheumatic fever. Circulation. **14:** 1138.
17. Cavelti, P. A. 1947. Experimental production of autoantibodies to heart, skeletal muscle, and connective tissue. A.M.A. Arch. Pathol. **44:** 1.
18. Cavelti, P. A. 1947. Cardiac lesions produced in rats by means of autoantibodies to heart and connective tissue. A.M.A. Arch. Pathol. **44:** 13.
19. Jaffe, R. & E. Holz. 1947–1948. Experimental allergic myocarditis. Exptl. Med. Surg. **5–6:** 189.
20. Maekawa, M. 1951. Allergie und immunität. First International Congress for Allergy, Zurich. Karger. Basel, Switzerland.
21. Shoji, H. 1955. Studies on the effective fractions isolated from myocardium cephalin as a determinant group of an antigen in heart allergy. Japan. Circulation J. **19:** 327.
22. Frick, E. 1951. Tier experimentelle untersuchungen über eine tuberkulos-allergische myokarditis. Z. ges. exptl. med. **117:** 393.
23. Kaplan, M. H. 1958. Immunologic studies of heart tissue. I. Production in rabbits of antibodies reactive with an autologous myocardial antigen following immunization with heterologous heart tissue. J. Immunol. **80:** 254.
24. Kaplan, M. H. 1958. Immunologic studies of heart tissue. II. Differentiation of a myocardial sarcoplasmic antigen and cardiolipin. J. Immunol. **80:** 268.
25. Kaplan, M. H. Unpublished observations.
26. Fischel, E. E. & R. H. Pauli. 1949. Serologic studies in rheumatic fever. J. Exptl. Med. **89:** 669.
27. Rejholec, V. & V. Wagner. 1955. Antimyocardial antibodies in rheumatic fever. Experientia (Basel). **11:** 278.
28. Osler, A. G., P. H. Hardy & J. T. Sharp. 1954. Fixation of complement by human sera and alcoholic extracts of human cardiac tissue. Am. J. Syphilis. **38:** 554.
29. Butler, K. & S. Moeschlin. 1956. Antikorper gegen verschiedene gewebszellen bei rheumatischen und anderen erkrankungen. Helv. Med. Acta. **23:** 592.
30. Steffen, C. 1958. Nachweis und wirkung von gewebs autoantikorpern bei primar-chronischer polyarthritis im vergleich mit anderweitig auftretenden gewebs-autoantikorpern. Immunopathologie I. Internationales Symposium, Basel/Seelisberg. : 376. Benno Schwabe. Basel, Switzerland.

31. Murphy, G. E. 1959. On muscle cells, Aschoff bodies and cardiac failure in rheumatic heart disease. Bull. N. Y. Acad. Med. **35:** 619.
32. Franklin, E. C. & H. G. Kunkel. 1957. Immunologic differences between the 19S and 7S components of normal human gamma globulin. J. Immunol. **78:** 11.
33. Dixon, F. J., J. J. Vazquez, W. O. Weigle & C. G. Cochrane. 1959. Immunology and pathogenesis of experimental serum sickness. *In* Cellular and Humoral Aspects of the Hypersensitive States. : 354. H. S. Lawrence, Ed. Hoeber. New York, N. Y.
34. Kaplan, M. H. 1959. The fluorescent antibody technic as a research tool in the study of connective tissue diseases. Arthritis and Rheumatism. **2:** 568.
35. Janton, O. H., R. P. Glover, T. J. E. O'Neill, J. E. Gregory & G. F. Froio. 1952. Results of the surgical treatment for mitral stenosis; analysis of 100 consecutive cases. Circulation. **6:** 321.
36. Soloff, L. A., J. Zatuchin, O. H. Janton, T. J. E. O'Neill & R. P. Glover. 1953. Reactivation of rheumatic fever following mitral commissurotomy. Circulation. **8:** 481.
37. Elster, S. K., H. F. Wood & R. Seely. 1954. Clinical and laboratory manifestations of the post-commissurotomy syndrome. Am. J. Med. **17:** 826.
38. Dressler, W. 1959. Post-myocardial infarction syndrome. A.M.A. Arch. Internal Med. **103:** 28.
39. Dresdale, D. T., C. B. Ripstein, S. V. Guzman & M. A. Greene. 1956. Post-cardiotomy syndrome in patients with rheumatic heart disease. Clinical evaluation and results with cortisone as a prophylactic and therapeutic agent. Am. J. Med. **21:** 57.

Discussion

Donald F. B. Char (*Department of Pathology, School of Medicine, University of Washington, Seattle, Wash.*): Experimentally produced cardiac lesions have been studied extensively in many laboratory animals. In view of the proved relationship of the group A beta hemolytic streptococci to the characteristic tissue alterations of rheumatic fever, it is inevitable that a vast amount of experimentation has been recorded in attempts to produce rheumatic carditis in lower animals. Various degrees of success have been claimed by investigators in their ability to reproduce the cardiac lesions of rheumatic fever.

The greater part of this work has been based on first establishing a state of hypersensitivity by the injection of suitable antigens. However, there is additional evidence that similar lesions may be produced in animals in the absence of hypersensitivity. There is little doubt that these cardiac lesions are frequently similar to some of the structural changes noted in the evolution of rheumatic fever.

There is a wealth of clinical data available in the post-mortem examinations of hearts of patients dying from known group A streptococcal infections, particularly scarlet fever, and it is most interesting to note that they have most of the features that many of the experimenters have claimed were pathognomonic of the tissue alterations of rheumatic fever. There is additional evidence to support the hypothesis that these acute cardiac lesions in known streptococcal infections are not synonymous with the Aschoff lesions of rheumatic fever in man.

We have employed group A streptococcal as well as nonstreptococcal substances in producing acute changes in the hearts of young rabbits. The Group A streptococcal material was prepared as filtrates of sonically disrupted bacteria. Single injections of these agents as well as multiple injections of these same materials produce cardiac damage; the latter do so in the absence of circulating antibodies. We can produce extensive cardiac damage with single injections of large doses of group A streptococcal materials that are as well

marked as those produced by the generalized Shwartzman reaction employing the same materials. The lesions we have produced are similar to those of other investigators who use the same or different materials.

By employing a variety of histochemical and immunochemical techniques and comparing the results to similar observations of human rheumatic carditis as well as scarlet fever myocarditis, the relationship between the experimental changes and the naturally occurring diseases may be demonstrated. It would seem that the experimental production of certain types of cardiac damage in laboratory animals is not a difficult problem. However, great caution must be exercised in attempting to extrapolate the results to a specific human disease such as rheumatic carditis.

STUDIES IN RHEUMATIC FEVER: III. HISTOCHEMICAL REACTIVITY OF THE ASCHOFF BODY*

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The Aschoff body continues to be the tissue hallmark of rheumatic carditis. Our knowledge of this structure is based largely on accumulated series of postmortem observations. Detailed morphologic studies in fatal cases of rheumatic carditis, when correlated with the clinical course, have yielded data relating "clinical" and "tissue" activity.[1] With the advent of cardiac surgery for the correction of rheumatic mitral valvular disease, biopsy material became available for histological analysis. These *intra vitam* tissues provided an unusual opportunity to study the rheumatic process in a dynamic manner.

Since 1954, our laboratory has studied 812 biopsies of the left auricular appendage obtained during mitral valvotomy. In this series, 23 per cent demonstrated Aschoff bodies in various stages of evolution. However, in only 16 cases were the classic histological criteria fulfilled for the tissue diagnosis of "acute rheumatic carditis." In all cases, preoperative clinical diagnosis was "chronic, inactive rheumatic mitral valvular disease." The clinical significance of the tissue findings has been discussed in previous publications.[2,3,4] The purpose of this paper is to present a detailed histochemical and immunochemical study of the Aschoff body in an attempt to define this pathognomonic structure by its chemical reactivity.

Materials and Methods

A total of 268 left auricular appendage biopsies was prepared for special study; the remaining 544 specimens had been fixed in 10 per cent formalin and processed in a routine manner. The tissues were received in the laboratory within 10 minutes after removal from the heart. After bisecting the tissue along its longitudinal axis, one half of the specimen was cut into small squares and quick-frozen in liquid nitrogen-chilled isopentane. The frozen samples were transferred to dry, rubber-capped glass bottles and immediately stored at −70° C. The other half of the original biopsy was subdivided, and samples were fixed in cold absolute acetone, cold buffered neutral formalin, and modified Carnoy solution (3 parts absolute alcohol:1 part glacial acetic acid). The frozen tissues were cut in a cryostat at −15 to −18° C. All fixed samples were paraffin-embedded and serially sectioned at 5 μ. Fluorescence microscopy was carried out utilizing a Leitz UAM microscope and a water-cooled GE-AH6 light source. Filter systems were based on the type of fluorescent dye used. For fluorescein isothiocyanate-labeled proteins, the techniques of Vazquez and Dixon[5] were employed. When lissamine rhodamine RB 200 was the fluor, the methods of Chadwick *et al.*[6] were used. The histochemical methods used have been described in previous publications from this laboratory.[7-12] The enzyme digestion

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studies and methods of optical quantitation have been standardized for analysis of fibrinoid as indicated in previous reports.[10]

Results

The Aschoff body: definition. Controversy continues to exist as to whether the lesions observed in the endocardium of these biopsy specimens are really Aschoff bodies. The classic descriptions of the Aschoff body were reviewed,

TABLE 1
TISSUES STUDIED IN ANALYSIS OF ASCHOFF BODY

Material	Anatomic-histological	Histochemical
Fulminating, fatal acute rheumatic carditis*	42	6
Chronic rheumatic heart disease, death due to congestive failure	30	3
Subacute bacterial endocarditis complicating rheumatic heart disease	8	4
Death in the postoperative period following mitral valvotomy	26	16
Left auricular appendage biopsies	812	268

* Made available from the collection of Rachel Ash during the period 1926 to 1936.

TABLE 2
HISTOLOGICAL CRITERIA USED FOR THE IDENTIFICATION OF THE ASCHOFF BODY

Connective tissue:
Focal swelling and fragmentation of collagen fibers.
Fibrinoid may or may not be present.
Elastic fibers usually not involved.
Cells:
Anitschkow cells usually present.
Aschoff cells: serrated, intranuclear, bar-shaped chromatin; basophilic, ragged, vague cytoplasm.
Lymphocytes frequently present.
Plasma cells may be present.
Inflammation:
Polymorphonuclear leukocytes: in acute cases.
Lymphocytes, plasma cells: variable degree of infiltration, usually focal (chronic active?, recurrent?)

and a study similar to that of Gross and Ehrlich was made. TABLE 1 indicates the tissues studied. In 1934, Gross and Ehrlich[1] stated that much of the confusion in the descriptive literature up to that date was due to the fact that different stages in the life cycle of the lesion were emphasized by various authors as the "typical Aschoff body." These authors further stated that the endocardial and subendocardial Aschoff bodies were usually compressed and elongated. The Aschoff cells were scattered throughout swollen collagen fibers and, if the collagen fibers were dense and compact, the cells would be irregularly compressed and assume bizarre polymorphous shapes. Saphir[13] again has called attention to the need for rigorous criteria in identifying the Aschoff body in human tissues.

Thus it is of importance to state clearly at the outset what is under consideration in this paper when the term Aschoff body is used. TABLE 2 states the structural criteria for the identification of this lesion, which are in full agree-

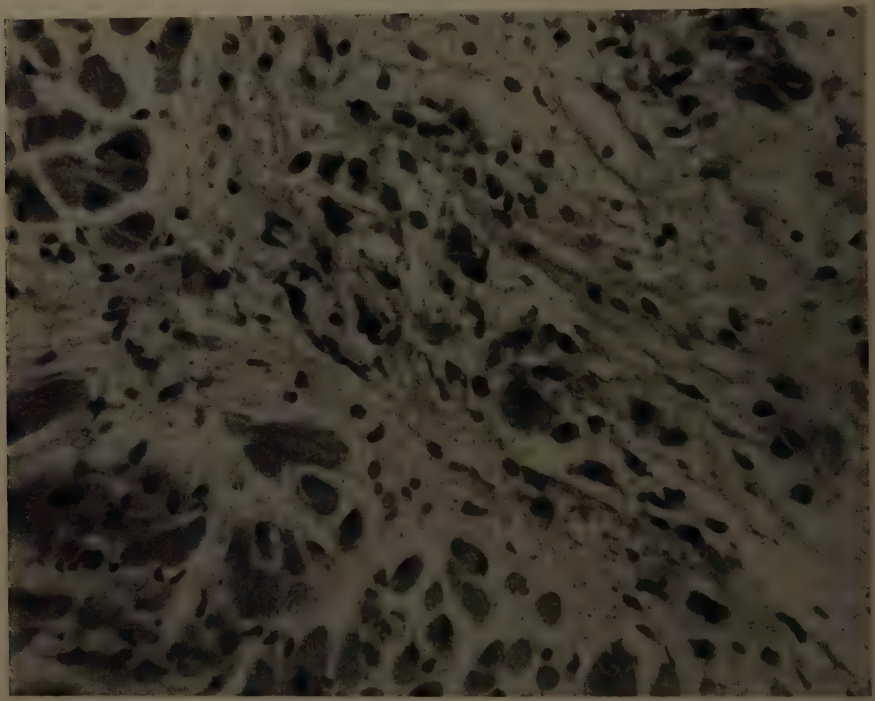

FIGURE 1. A typical endocardial Aschoff body in a left auricular appendage biopsy H and E. ×175.

TABLE 3
CLASSIFICATION OF AURICULAR APPENDAGE BIOPSIES

	Routine study	Histochemical study
Acute rheumatic carditis	16	9
Aschoff bodies present	171	78
Nonspecific changes	625	181
	812	268

ment with those of the majority of workers in this field. The results of the histochemical studies are based almost entirely on the biopsy specimens (TABLE 3). FIGURE 1 is a typical example of the lesions studied.

Connective Tissue Changes

In almost every Aschoff body studied the collagen fibers in and immediately around the lesion showed structural changes. These were best seen with the

van Gieson and aniline blue-chromotrope 2R staining methods. The collagen fibers were fragmented and swollen with variable degrees of compactness. FIGURE 2 shows the collagen fiber alterations with an increase in interfibrillar ground substance. Collagen fiber changes were observed as focal lesions and, on serial section examinations, these altered fibers blended with normal fibers once beyond the limits of the Aschoff body. Attempts were made to utilize preparations of collagenase to digest the fibers as a histochemical method without success. Elastic fiber stains and silver methods showed the elastic fibers

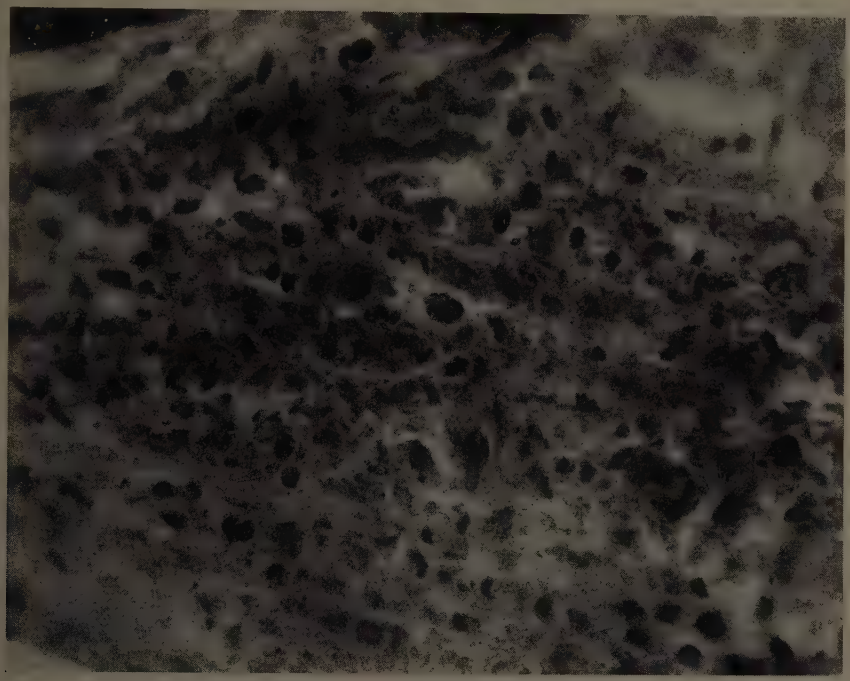

FIGURE 2. Region of an Aschoff body without nuclear counterstain. Note the swollen, fused, and fragmented collagen fibers. Fibrinoid material present. Aniline blue-chromotrope 2R. ×175.

to be essentially intact. Faintly staining, short argyrophilic fibers could be identified especially if fibrinoid material was present. Elastic fibers also could be visualized by their intense autofluorescence.

In almost all cases, increased acid mucopolysaccharides of the ground substance could be demonstrated primarily in the central area of the Aschoff body and also in the periphery. TABLE 4 indicates the methods used to characterize the acid mucopolysaccharides. The intensity of the reactions decreased as fibrosis or repair increased. FIGURE 3 shows an area of fibrinoid staining intensely metachromatic with toluidine blue. The positive histochemical and immunohistochemical reactions of the ground substance were increased in intensity when fibrinoid was present. The reactivity of the acid mucopolysaccharides strongly suggests that they are of the sulfated type. Metachromasia

was frequently present in the absence of fibrinoid, and the reaction could be blocked effectively by the action of testicular hyaluronidase. The Alcian blue and colloidal iron reactions closely paralleled the demonstration of metachromasia. The testicular hyaluronidase preparation used demonstrated chrondromucinase activity as well.

The histochemical reactions for various general types of proteins, reactive groups, and two amino acids (TABLE 4) in the ground substance varied with the intensity of the lesion. Developing Aschoff bodies (TABLE 5) demonstrated the most intense reactions, which then decreased as the mature structure evolved. When the Aschoff body began to show signs of healing, as noted by decrease in number of cells with wide scatter and sclerosis, these reactions diminished

TABLE 4

HISTOCHEMICAL ANALYSIS OF GROUND SUBSTANCE IN THE ASCHOFF BODY

Mucopolysaccharides:	
Toluidine blue, aq. *p*H 4.5	metachromasia + to +++
Sulfation-metachromasia	slight increase
Streptococcal hyaluronidase digestion	no effect
Testicular hyaluronidase digestion	complete inhibition of metachromasia
Schiff reaction	no free aldehyde groups
Periodic acid-Schiff reaction (PAS)	++ to +++
Periodic acid-phenylhydrazine-Schiff	no reaction, aldehydes blocked
Alcian blue-chromotrope 2R	++ to +++
Colloidal iron—PAS	++ to +++
β-Glucuronidase, diastase, collagenase	no specific effects
Proteins:	
DNA (Feulgen method)	−
RNA (methyl green-pyronine, Azure A-ribonuclease)	usually negative, except in acute carditis + to ++
Basic proteins	− to +
Protein-bound amino groups	− to ±
Protein-bound SH and SS groups	− to ++
Tyrosine	+ to ++
Tryptophane	+ to ++
Lipids:	
Neutral fat	−
Phospholipids	+ to ++

Key: + to +++, intensity of positive reaction; −, no reaction.

markedly in their intensity and frequency. The demonstration of phospholipids also correlated with the early phases in the life cycle of the lesion.

In addition to the histochemical procedures for proteins, the immunohistochemical methods as listed in TABLE 6 were of value. Gamma globulin was regularly localized in varying intensity. Localization was usually in the connective tissue in and around the Aschoff body, with frequent streaks in the adjacent areas. Fibrin was localized infrequently and with questionable intensity. An interesting observation was the more frequent localization of fibrin in fibrinoid. However, this localization never reached the intensity and frequency of gamma globulin. Attempts to localize Group A streptococcal protein in the region of the Aschoff body were not successful. The application of directly labeled streptococcal sonicated proteins and streptolysin O in an attempt to demonstrate antibodies to these substances was also unsuccessful.

FIGURE 3. Tangential section of endocardial Aschoff body. Intense metachromasia of fibrinoid and adjacent substance. Toluidine blue. ×300.

The response of fibrinoid to various enzymes as shown in TABLE 7 suggests that this material is rich in proteins and acid mucopolysaccharides. The ability of plasmin to alter the fibrinoid deposits significantly was quite variable and more pronounced in the post-mortem studies. This result is under further investigation. Previous studies have shown that the Aschoff bodies in the endocardium of the left auricular appendage and in the ventricular stroma are

TABLE 5
HISTOCHEMICAL ANALYSIS OF ASCHOFF CELLS IN THE ASCHOFF BODY

	Positive results
Nucleus:	
DNA	++
Basic protein	++
Protein-bound amino groups	++
Cytoplasm:	
RNA	+++
Alpha-amino groups	+++
SH and SS groups	± to +
Tyrosine, tryptophane	++(?)
Acid mucopolysaccharide	+++
Acid phosphatase	+++
Alpha naphthyl esterase	+++
Aminopeptidase	++

Key: + to +++, intensity of positive reactions.

TABLE 6
IMMUNOHISTOCHEMICAL ANALYSIS OF ASCHOFF BODY

Fluor	Connective tissue	Fibrinoid
Rabbit antihuman γ globulin	+ to ++	+++
Rabbit antihuman fibrin	− to +	+ to ++
Rabbit antihuman albumin	− to +	− to ±
Rabbit antistreptococcal protein	−	−
Streptococcal sonicate	−	−
Streptolysin O	−	−
C-reactive protein	−	−

Key: + to +++, intensity of fluorescence. Rabbit antibodies labeled with fluorescein isothiocyanate,* other proteins labeled with lissamine rhodamine RB 200.†
* Sylvana Chemical Co., Orange, N. J.
† Imperial Chemical Industries, Manchester, England.

similar.[2,5,9] General topographical considerations, however, may influence the frequency, rate, and quantity of chemical substances present. FIGURE 4 shows the removal of fibrinoid by mild tryptic proteolysis.

The Aschoff cell, which is derived from the Anitschkow cell, was studied as shown in TABLE 5. The nuclear reactions for proteins were positive for DNA, basic proteins, and reactive amino groups. FIGURE 5 shows the typical serrated bar-shaped intranuclear chromatin of several Anitschow cells following the aniline blue-chromotrope 2R procedure. The same intranuclear pattern was observed in Aschoff cells.

The cytoplasm of the Aschoff cell gave an intense reaction for RNA and reactive alpha amino groups. The various reactions for connective tissue and fibrinoid acid mucopolysaccharides frequently showed staining of the Aschoff cell cytoplasm. This may represent diffusion artifact. FIGURE 6 shows the reaction for acid phosphatase using the Gomori technique.[12] Note the occasional nuclear reaction due to cytoplasmic diffusion. The reaction for esterase using alpha naphthyl acetate as a substrate and naphthanil diazo blue B as a coupling agent is shown in FIGURE 7.[12] There appears to be intense cytoplasmic activity with apparent nuclear involvement. Aminopeptidase activity was determined using L-leucyl-β-naphthylamide as a substrate and diazotized *o*-aminoazotoluene as the diazonium coupler.[14] FIGURE 8 shows this reaction without counterstain.

TABLE 7
SUSCEPTIBILITY OF FIBRINOID TO ENZYME ACTION

Enzyme	Aschoff bodies	
	Auricular appendages	Ventricular myocardium
Trypsin*	+++	+++
Pepsin*	+++	+++
Plasmin	− to ±	+
Strep. hyaluronidase	−	−
Test. hyaluronidase*	++	+
β-Glucuronidase*	−	−
Collagenase†	−	−

Key: + to +++, slight to complete digestion; −, no effect.
* Worthington Biochemical Corp., Freehold, N. J.
† Agricultural Biochemical Co., Lynbrook, N. Y.

Discussion

There can be little doubt that the lesions observed in surgically removed left auricular appendages are truly Aschoff bodies. Gross[15] clearly reviewed the various stages of this lesion in the left auricle. Reports from various parts of the world are in essential agreement with the established findings.[16,17,18] The clinical significance of these Aschoff bodies, however, has not been determined. In order to understand more fully the nature of this unique structure, the studies described were undertaken.

The observations confirm the widely held notion that collagen fiber damage is an essential component of the connective tissue changes in the evolution of the Aschoff body. Detailed studies of this point by various workers[12,13,16,17] further support this evidence, and it is difficult to understand Murphy's[18] statement that Aschoff bodies can develop without evidence of collagen changes.

The demonstration of increased acid mucopolysaccharides in the Aschoff body also has been reported by others.[17-19] However, the data obtained indicate that the mucopolysaccharides are highly sulfated and in association with proteins. This material may be related to the mucopolysaccharides of heart valve. Deiss and Leon[20] considered this to be a mixture of two chondroitin

FIGURE 4. Loss of fibrinoid following digestion with trypsin. Note that configuration of the Aschoff body and cells remain intact. Trypsin 0.5 mg./ml., *p*H 8.2, 37° C. for 90 min. H and E. ×175.

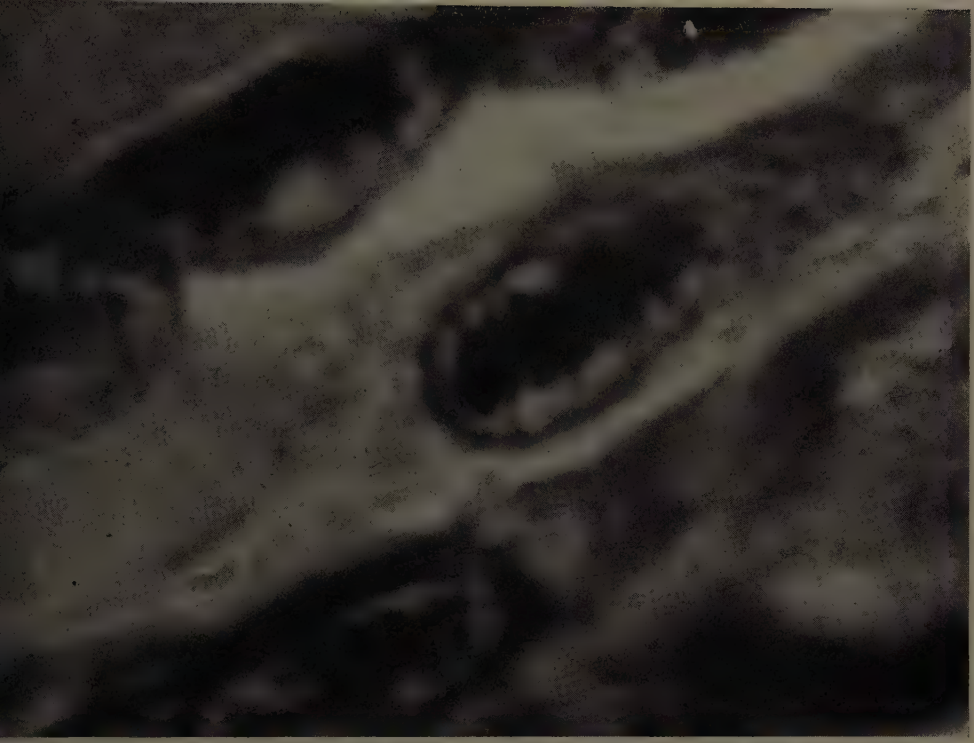

FIGURE 5. Anitschkow cells developing in connective tissue stroma adjacent to an Aschoff body. Note the distinct intranuclear chromatin pattern. The chromatin basic proteins stain intensely with chromotrope 2R. Aniline blue-chromotrope 2R. ×360.

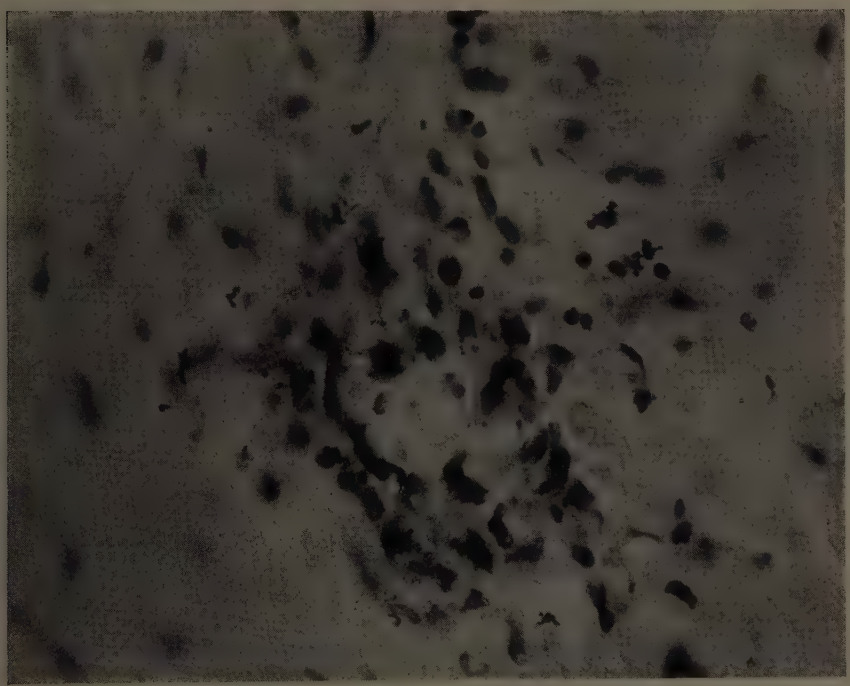

FIGURE 6. Acid phosphatase reaction (Gomori). Note the intense staining of the Aschoff cells with nuclear diffusion. Central area of fibrinoid. No counterstain. ×175.

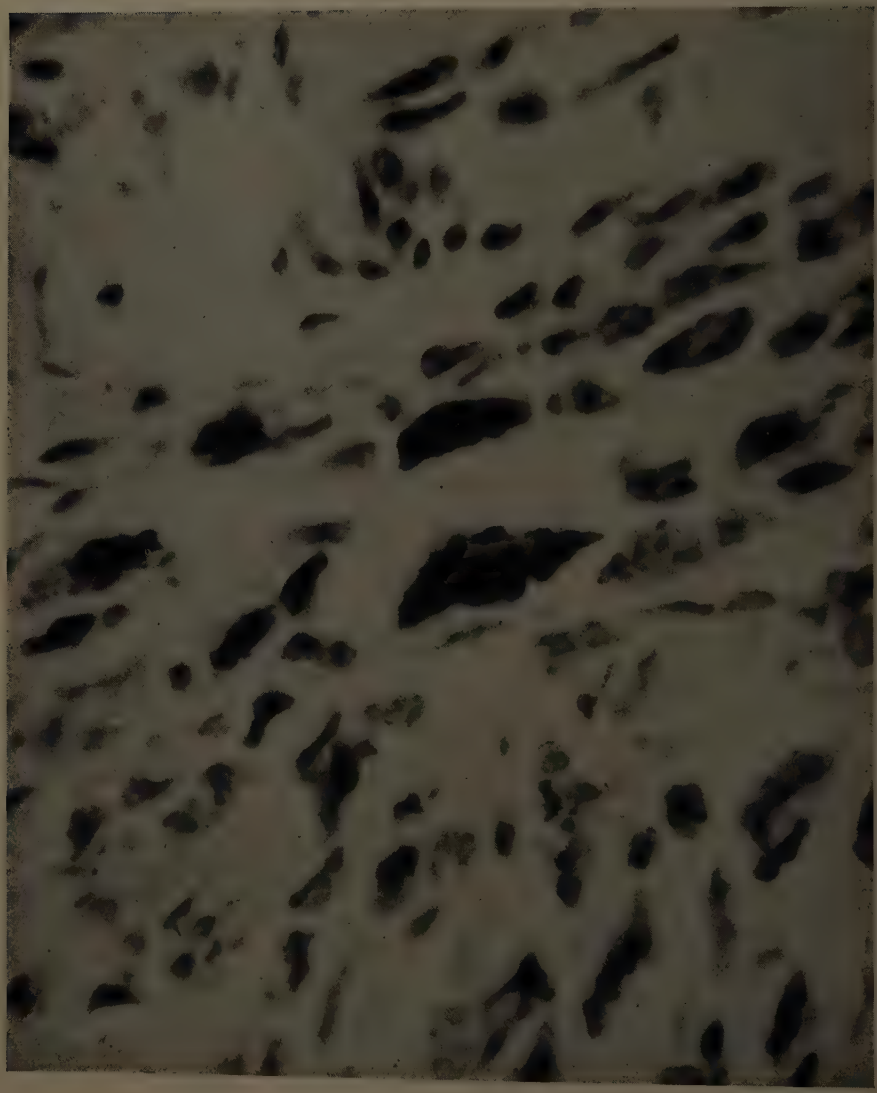

FIGURE 7. Esterase reaction using alpha-naphthyl acetate as substrate. Aschoff cells stain intensely, frequently obscuring nuclear detail. No counterstain. ×220.

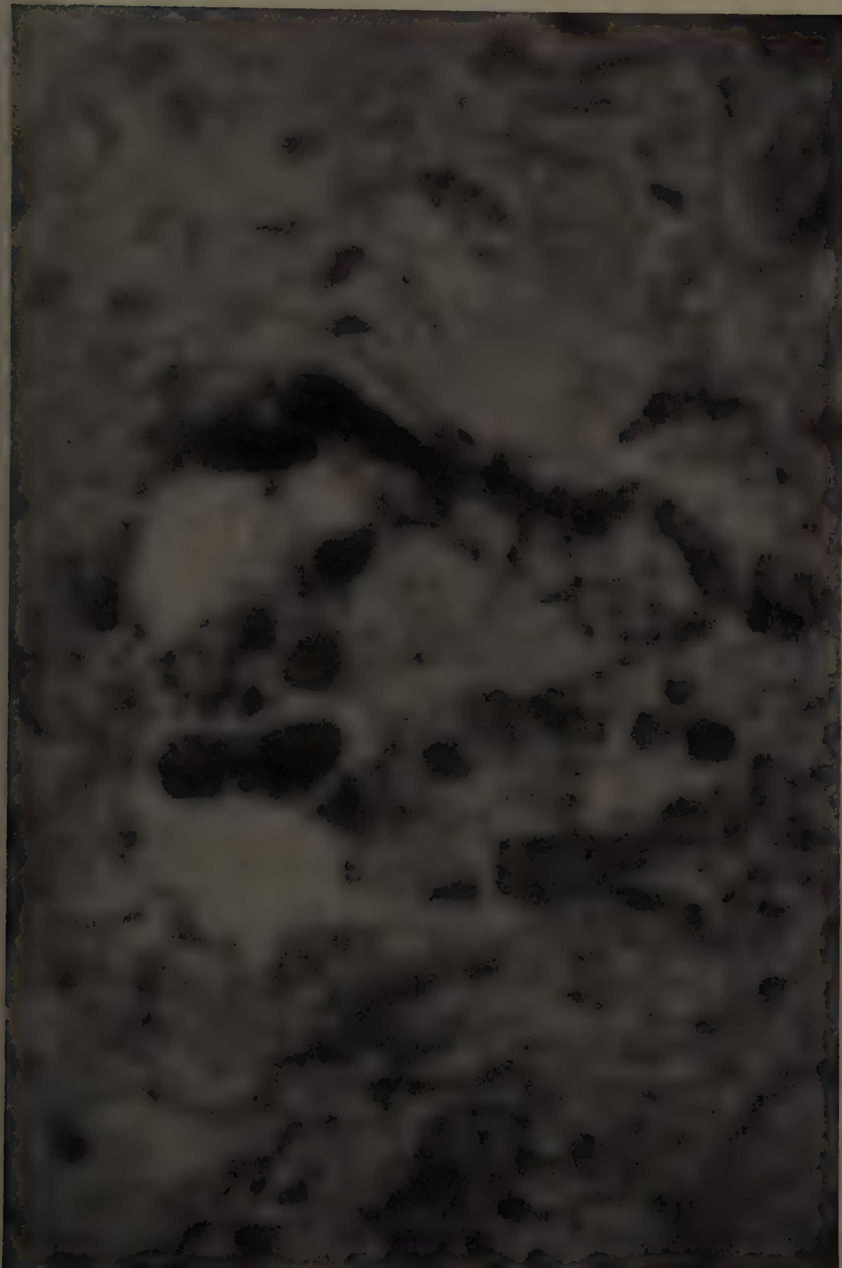

FIGURE 8. Aminopeptidase reaction (Burstone). Diffuse staining of Aschoff cell cytoplasm and some stromal reaction. No counterstain. ×300.

sulfates and hyaluronic acid. Strukov and Orlovskaya[17] consider the accumulation of acid mucopolysaccharides to be intimately associated with the breakdown of collagen to procollagen. We were unable to confirm their observations concerning the action of clostridial collagenase on the altered collagen in the Aschoff body.

The exact character of the proteins complexed with the mucopolysaccharides remains unknown. The observations in this paper show the presence of basic proteins with reactive alpha-amino, SH, and SS groups. Fluorescent antibody studies localize gamma globulin in this area. A composite picture emerges that might be visualized as follows: (1) simultaneous change in collagen fibers and ground substance, (2) focal fragmentation and swelling of collagen with increased chondroitin sulfates, and (3) proteins increased with specific identification of gamma globulin.

At this point rheumatic fibrinoid should be considered. While the above theoretical sequence of events may take place without fibrinoid, the presence of fibrinoid intensifies the alterations. Thus the fibrinoid in the Aschoff body appears to be derived from altered collagen, acid mucopolysaccharides, and proteins when the process is accelerated. Repeated attempts to define fibrin as a major component of this fibrinoid have been unsuccessful. Other investigators[5,17,20,21] have reported similar results, and the positive findings of fibrin by one group[22] are difficult to explain. The enzymatic digestion pattern of fibrinoid suggests that proteins constitute the significant portion. It is not possible to distinguish the gamma globulin localized from immune or serum globulin.

The origin of the Aschoff cell has been proposed from myofibers,[18] fibroblasts[23] and histiocytes.[7] In previous reports[2,7] we have demonstrated that on the basis of anatomical considerations the myofiber theory did not seem possible. The reactivity of the cytoplasm of the Aschoff cell clearly sets it apart from normal and degenerating cardiac myofibers. Fibroblasts in the vicinity of the Aschoff body do not demonstrate the histochemical reactions of the Aschoff cell. However, Anitschkow cells frequently show patterns similar to those of the Aschoff cell. The presence of cytoplasmic acid phosphatase and alpha naphthyl esterase in the Aschoff cell is similar to findings by Braunstein *et al.*[24] in lymph node histiocytes. Also, the studies of Gropp and Hupe[25] and Gusek[26] indicate the presence of these enzymes in macrophages. While no specific enzyme pattern is known for histiocytes, fibroblasts, and myofibers, nevertheless, the total observations strongly suggest that in the Aschoff body the Aschoff cell can be readily identified. It is tempting to speculate that the Aschoff cell represents a specialized mesenchymal cell of the histiocyte-macrophage type. This would then place the Anitschkow cell as a cardiac histiocyte, a term previously employed.

As a final structured tissue change, the Aschoff body fulfills the criteria for a granuloma. The histochemical studies confirm and extend this concept. Attempts were made to localize Group A streptococcal proteins and certain antistreptococcal antibodies in the Aschoff body. These studies were uniformly unsuccessful. A more detailed exploration of other streptococcal materials to the pathogenesis of the Aschoff body is under investigation.

A detailed review of the life cycle of the Aschoff body and the important

implications of the biopsy studies is beyond the scope of this paper. However, the histochemical data serve to extend the parameters of objective evaluation of the Aschoff body and allow for identification beyond classic morphology. Of equal significance is the need to standardize observations that will allow for the recognition of the Aschoff body in an experimental model. The application of histochemical procedures to experimentally induced carditis quickly reveals the lack of relationship to the human rheumatic process. Finally, the mechanisms relating Group A beta-hemolytic streptococci to the specific Aschoff lesion may now begin to be explored at a histochemical level in man and animals. Investigations in these directions are now in progress.

Summary

A detailed histochemical study of the Aschoff body as observed in 78 surgical biopsies and 9 post-mortem cases is presented. The earliest recognizable changes are simultaneous events involving collagen fibers, acid mucopolysaccharides, and proteins. The focal connective tissue changes are soon surrounded by Anitschkow cells and Aschoff cells. The highly characteristic intranuclear chromatin arrangement separates these cells from myofibers and fibroblasts. Enzymatic reactivity of the Aschoff cell cytoplasm suggests an origin from histiocytes. The Aschoff body is thus a true granuloma. Rheumatic fibrinoid represents a mixture of substances that vary with the age and intensity of the process. The localization of gamma globulin in the fibrinoid and connective tissue changes may be of significance. Attention is directed to the application of the data to the experimental production of rheumatic carditis in animals.

References

1. Gross, L. & J. C. Ehrlich. 1943. Studies on the myocardial Aschoff body. I. Descriptive classification of lesions. Am. J. Pathol. **10:** 467.
2. Tedeschi, C. G., B. M. Wagner & K. C. Pani. 1955. Studies in rheumatic fever. I. Clinical significance of the Aschoff body based on morphological observations. A.M.A. Arch. Pathol. **20:** 408.
3. Pani, K. C., B. M. Wagner & S. H. Shapiro. 1958. Serological, electrophoretic and histological observations in mitral commissurotomy patients. Am. J. Med. Sci. **236:** 590.
4. Tedeschi, C. G. & B. M. Wagner. 1956. The problem of subclinical rheumatic carditis. Am. J. Med. Sci. **231:** 382.
5. Vazquez, J. J. & F. J. Dixon. 1958. Immunohistochemical analysis of lesions associated with fibrinoid changes. A.M.A. Arch. Pathol. **66:** 504.
6. Chadwick, C. S., M. G. McEntegart & R. C. Nairn. 1958. Fluorescent protein tracers. Lancet. **8:** 412.
7. Wagner, B. M. & C. G. Tedeschi. 1955. Studies in rheumatic fever. II. Origin of cardiac giant cells. A.M.A. Arch. Pathol. **20:** 423.
8. Wagner, B. M. 1955. Histochemical studies of fibrinoid substances and other abnormal tissue proteins. I. Application of Roque's chromotrope stain. A.M.A. Arch. Pathol. **59:** 58.
9. Wagner, B. M. 1955. Histochemical studies of fibrinoid substances and other abnormal tissue proteins. II. Use of fibrinolytic enzymes. A.M.A. Arch. Pathol. **59:** 63.
10. Wagner, B. M. 1957. Histochemical studies of fibrinoid substances and other abnormal tissue proteins. III. Proteolysis of fibrinoids. J. Mt. Sinai Hosp. **24:** 1323.
11. Wagner, B. M. 1957. Hypersensitivity: role of the connective tissue. *In* Analytical Pathology. : 429–470. R. C. Mellors, Ed. McGraw-Hill. New York, N. Y.
12. Wagner, B. M. 1958. Studies on the pathogenesis of the Aschoff body. Circulation (Part II). **18:** 791.
13. Saphir, O. 1959. The Aschoff nodule. Am. J. Clin. Pathol. **31:** 534.

14. Burstone, M. S. 1956. Histochemical demonstration of proteolytic activity in human neoplasms. J. Natl. Cancer Inst. **16:** 1149.
15. Gross, L. 1935. Lesions of the left auricle in rheumatic fever. Am. J. Pathol. **11:** 711.
16. Stojalowski, K. & A. Krygier. 1955. Histochemical and microscopic studies in rheumatism. Pediatrics. **30:** (Suppl. 11). Pol.
17. Strukov, A. I. & G. V. Orlovskaya. 1957. Histochemical investigation of connective tissue in cases of acute rheumatism. Ann. Rheumatism Disease. **16:** 307.
18. Murphy, G. E. 1959. On muscle cells, Aschoff bodies and cardiac failure in rheumatic heart disease. Bull. N. Y. Acad. Med. **35:** 620.
19. Horn, V. 1957. Veränderungen der Bindegewebegrundsubstanz in den Amputierten Herzohren bei Mitralstenosen. Zentr. allgem. Pathol. u. pathol. Anat. **97:** 280.
20. Deiss, W. P. & A. S. Leon. 1955. Mucopolysaccharides of heart valve mucoprotein. J. Biol. Chem. **215:** 685.
21. Lannigan, R. 1959. The rheumatic process in the left auricular appendage. J. Pathol. Bacteriol. **77:** 49.
22. Brux, J. De. 1958. Role of globulin constituents of fibrinoid in histogenesis and classification of so-called collagen diseases. Presse méd. **66:** 661.
23. Costero, I., R. Barroso-Moguel, A. Chevez, G. Monroy & R. Contreras. 1958. Rheumatic fever lesions in patients treated with cortisone. I. Special study of endomyocarditis. Arch. inst. cardiol. Méx. **28:** 155.
24. Braunstein, H., D. G. Freiman & E. A. Gall. 1958. A histochemical study of the enzymatic activity of lymph nodes. Cancer. **11:** 829.
25. Gropp, A. & W. S. Hupe. 1958. Nachweis von Enzymen an *in vitro* Gezüchteten Makraphagen und Epitheloiden Zellen. Arch. pathol. Anat. u. Physiol. Virchow's. **331:** 641.
26. Gusek, W. 1958. Die Feinstruktur der Einkernigen Makraphagen und der Mehrkernigen Riezenzellen im Frendkorpergranulations gewebe. Frank. Z. Pathol. **69:** 429.

Discussion

Joseph C. Ehrlich (*Director of Laboratories, Lebanon Hospital, New York, N.Y.*): Consideration of cardiac lipofuscin, the normally occurring intracellular pigment of heart muscle, has some bearing on the problem of the origin of the constituent cells of Aschoff bodies. It has been argued that structural details of cardiac myofibers disappear as a result of degeneration, and that therefore the absence of cross striations and myofibrillae in the cells of Aschoff bodies is not inconsistent with their origin from muscle. This argument does not hold, however, for lipofuscin. Lipofuscin granules resist degenerative processes that destroy other constituents of cardiac muscle cells. In this respect cardiac lipofuscin is similar to hepatic lipofuscin. The latter is known to resist severe hepatocellular necrosis such as occurs, for example, in acute viral hepatitis; even after total destruction of parenchymal cells, lipofuscin pigment released by the dead cells is picked up by Kupffer cells and other phagocytes. Similarly, in the heart, after infarction with necrosis of myofibers, lipofuscin pigment survives and may be demonstrated in phagocytes (including "caterpillar" cells) in the stroma in the vicinity of infarcts. The failure to find lipofuscin in Aschoff cells must be explained by proponents of the musculogenic theory of the origin of these cells.

Another point concerns rather old-fashioned histochemistry. Degenerating cardiac muscle fibers contain sudanophilic lipid. Fine droplets at first and coarse fat droplets later, accumulate characteristically within the cytoplasm. Sudan stains of frozen sections of hearts with large numbers of Aschoff bodies in an early stage of evolution often reveal droplets of sudanophilic lipid in scattered groups of muscle fibers undergoing degenerative changes, but none in Aschoff body cells. This is another fact to be considered in assessing the role, if any, of degenerating muscle cells in the formation of Aschoff bodies.

With regard to the occurrence of smooth muscle in heart valves, it should be emphasized that this muscle is confined to the endocardial covering of the valves. Its occurrence there is not an adequate explanation of the genesis of Aschoff cells, because Aschoff bodies are found not only in the endocardium but also in the muscle-free fibrous cores of heart valves. Regarding the descent of auricular myocardium into valves, this occurs for a short distance only, and

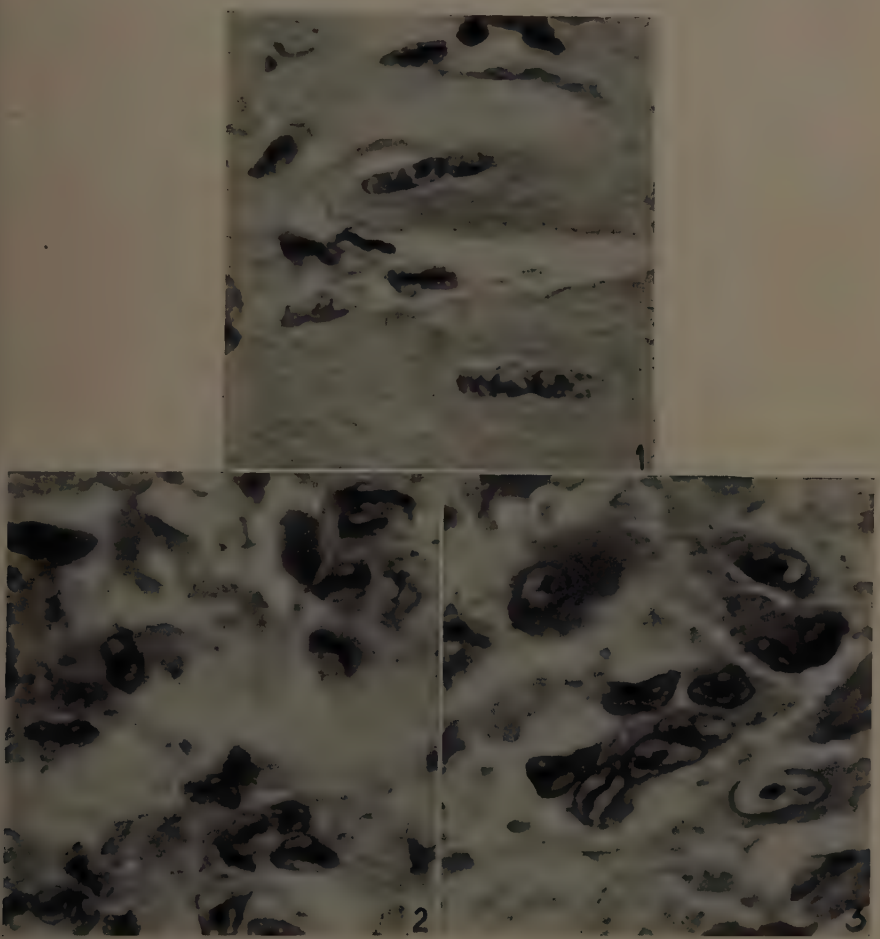

FIGURE 1. Normal appearance of nuclei of cardiac muscle cells and "caterpillar" cells. ×540.

FIGURE 2. Aschoff body in early stage of evolution. Nuclei of Aschoff body cells contain intranuclear axial structures attesting their derivation from "caterpillar" cells. ×540. (No. A910, Barnert Memorial Hospital, Paterson, N. J. Age, 7 years; first attack of rheumatic fever; ill 6 days prior to admission; died day of admission. Courtesy Jacob Churg.)

FIGURE 3. Multinucleated giant cell in Aschoff body. The intranuclear axial body in one of the nuclei of this giant cell is longitudinally sectioned, attesting its "caterpillar" cell source. Others are tangentially sectioned, creating the so-called "owl's eye" pattern. ×540. (No. 5102, Paterson General Hospital, Paterson, N. J. Age, 22 months; first attack of rheumatic fever; ill 7 to 10 days prior to admission; died day of admission. Courtesy Herbert Cole.)

chiefly in the anterior leaflet of the mitral valve. The auricular muscle wedge does not descend normally into the posterior leaflet of the mitral valve, a leaflet that commonly contains Aschoff bodies. Similarly, striated muscle does not occur in the semilunar valves, where Aschoff bodies also may be observed.

In the last analysis, however, the critical consideration regarding the genesis of Aschoff cells has to do with intranuclear morphology—a point that has been somewhat neglected. Cases of rheumatic carditis with Aschoff bodies, in which there have been repeated attacks or in which the activity, whatever that term may mean, has persisted for some time, are not suitable for the study of intranuclear morphology of Aschoff cells because of changes that occur as the Aschoff body itself ages. More suitable are those cases, preferably of very young persons, in which there is no clinical or anatomic evidence of previous attacks, in which the total clinical duration of the disease is very short (a matter of days or weeks), and concerning which there is every reason to believe that the Aschoff bodies are in an early stage of their evolution. In such hearts, the intranuclear morphology of the Aschoff cells is easily and characteristically traceable to the normally occurring "caterpillar" cells, or so-called Anitschkow cells. The axial intranuclear body of the caterpillar cells is so structurally distinctive that it cannot be confused with cardiac muscle cells. The occurrence of these distinctive bodies in the nuclei of Aschoff cells identifies them at once as derivatives of caterpillar cells (FIGURES 1, 2, and 3). Cardiac giant cells of muscular origin are well known because of experience with ordinary myocardial infarcts. Such giant cells may or may not contain residual cross striations. However, their nuclei retain intranuclear morphologic characteristics similar to nuclei of normal cardiac muscle cells and completely different from the nuclei of normal caterpillar cells and Aschoff cells.[1,2,3]

References

1. EHRLICH, J. C. & B. LAPAN. 1939. The Anitschkow "myocyte." A.M.A. Arch. Pathol. **28:** 361–370.
2. CLAWSON, B. J. 1941. Relation of the "Anitschkow myocyte" to rheumatic inflammation. A.M.A. Arch. Pathol. **32:** 760–763.
3. SAPHIR, O. 1959. The Aschoff nodule. Am. J. Clin. Path. **31:** 534–539.

THE CARDIAC EFFECTS OF GROUP A STREPTOCOCCAL SONICATES IN RABBITS*

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The accumulated data show an overwhelming relationship between group A streptococci and rheumatic fever. However, the exact nature of this relationship in the pathogenesis of the disease remains obscure.[1-3] There are currently two major theories regarding the role of this organism in provoking the cardiac changes characteristic of rheumatic fever. These hypotheses relate the disease either to a hypersensitivity mechanism or to the direct action of biologically active materials. In an attempt to elucidate this problem, many investigators have tried to reproduce the pathological process in animals. A variety of experimental systems has been devised utilizing the following agents: (1) horse, bovine, duck, and pig sera, and fractions thereof;[4-8] (2) living and killed streptococci and various fractions and products thereof;[9-13] (3) various materials from patients suffering from rheumatic fever;[14-15] (4) combinations of normal organ extracts and the above-listed materials;[16] and (5) histamine, mineralocorticoids, various Gram-negative endotoxins such as *Escherichia coli* lipopolysaccharide and meningococcal endotoxin.[17-21]

Although the direct acute effects of these agents have been studied, most experiments have employed the delayed hypersensitivity or anaphylactic shock mechanism. The majority of the experiments and observations involved the myocardial changes noted in rabbits,[21] but other animals have been employed, including the guinea pig,[22] rat,[16] mouse,[12] monkey,[11] swine,[20] and dog.[23]

Since 1904, when Aschoff[24] clearly stated that the granuloma of rheumatic fever was unique and specific, this pathognomonic concept has been accepted. Proof of rheumatic fever in nonhuman hosts therefore would depend largely on the demonstration of Aschoff bodies in the heart. Various workers have claimed that they have reproduced aspects of the tissue changes characteristic of rheumatic carditis in lower animals. Attempts to confirm these observations and a critical re-evaluation of the data have led us and others[25,26] to conclude that the Aschoff body has not been noted in animals studied thus far. The paper by Bernard Wagner in this monograph confirms and extends previously held notions concerning the nature of the Aschoff body.

In 1957, Schwab and Cromartie[27] described a previously unknown toxic cellular component of group A streptococci that produced chronic remittent skin lesions in rabbits upon intradermal injection, suggesting that it had a specific biological effect on connective tissue. Since the earliest morphologic alteration observed in the evolution of the Aschoff body is in the connective tissue, this material prepared from sonicated cultures of group A streptococci was of great interest. In addition, the sonicates represented a close approximation of intact but nonviable streptococci. The systemic administration of this material to rabbits seemed to be worthy of further study.

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The purpose of this paper is to present the findings in rabbits following the administration of group A streptococcal sonicates. Special attention has been paid to the cardiac changes and their significance as regards specificity.

Methods and Materials

New Zealand white male rabbits, weighing 2 to 3 kg. each, were maintained on a standard ad libitum Purina rabbit pellet ration.

The type I group A streptococcus was obtained from the laboratory of William Cromartie; the types VI and XII streptococci were obtained from Russell Weiser.

The cultivation, harvesting, and extraction of the organisms were carried out according to the directions of Schwab.[28] Washed cells from 18-hour cultures of streptococci in Todd-Hewitt beef heart broth were suspended in phosphate buffer solution (*p*H 7.0, 0.15 *M*), subjected to ultrasonic oscillations for 45 to 95 min., then filtered through a Chamberland L3 filter. The filtrate was routinely tested for sterility by subculturing on blood agar plates, and then was stored at −20° C. in sealed ampules until used. Nitrogen content of each batch of filtrate was determined by the Kjeldahl method.

The streptococcal broth filtrate was prepared by filtering broth in which type XII streptococcus was grown through a Chamberland L3 filter and was tested for sterility before use in the experiments.

The bovine serum albumin was obtained from a commercial source,* and the human serum was derived from a healthy worker in our laboratory.

Conjugation with lissamine rhodamine RB200 was done according to the direction of Chadwick *et al.*[29] The conjugated serum proteins served as nonspecific controls in the experiment. Detailed studies of the tissue distribution of rhodamine-labeled serum proteins and streptococcal sonicate are beyond the scope of this paper and will be presented elsewhere.

The Freund's-type adjuvant employed in some of the experiments contained the *Mycobacterium butyricum* and was prepared in our laboratories.

In all experiments studying the acute effects of the agent employed, the materials were injected intravenously via the marginal ear veins, and the animals were observed daily until sacrificed 48 hours after the injection. In the 2-dose experiments, the agents were injected initially as above, injected again 24 hours later, and the animals were sacrificed at 48 hours. These experiments are similar in design to those of Thomas *et al.*[21]

In the long-term experiments, the initial injection of the type I streptococcal sonicate in Freund's adjuvant was given, then 3 subsequent intravenous injections of the same type of streptococcal sonicate without adjuvant were administered at monthly intervals. Sacrifice of the animals occurred at 120 days after the first injection, or 30 days after the last injection of the materials.

The dose of the streptococcal sonicate was 2 mg. of nitrogen per kilogram of body weight per injection, except where indicated otherwise in the test. All animals were sacrificed by the intravenous administration of Nembutal, except for 1 rabbit that died spontaneously during the experimentation. A complete autopsy was routinely performed. The thoracic organs were removed *in toto*

* Armour and Company, Chicago, Ill.

and the thymus and lungs were carefully dissected. The heart was opened, following the flow of blood. After washing, the structures were carefully examined under a hand lens. Routine sections of heart included: anterior mitral leaflet with attached atrium, chordae tendinae, and ventricular myocardium, posterior mitral leaflet with attached atrium, interventricular septum including aortic valve and annulus, tricuspid valve leaflet with attached right atrium (edge of coronary sinus orifice), and right ventricular myocardium. Sections were taken to include coronary arteries. In addition, representative samples of kidney, spleen, lungs, adrenals, and liver were taken. Appropriate sections were fixed in cold buffered neutral formalin and cold acetone. When indicated, tissues were quick-frozen and stored at $-20°$ C. The staining reactions and histochemical procedures used in this laboratory for connective tissue study have been described (see paper by Wagner, this monograph).

Blood specimens were taken routinely at the time of sacrifice and tested for evidence of antibodies by the ring precipitin technique. The tests were completely negative in the acute experiments, but were markedly positive in the long-term repeated injection series.

A total of 21 animals remained untreated and were sacrificed whenever an experiment was terminated. These animals served as controls for that group, so that we were certain that no epizootic type of infection, as reported by Miller[30] and Loewe and Lenke,[31] was present in our rabbits. Moreover, prior experience with stock New Zealand male rabbits had demonstrated a spontaneous incidence of mild focal myocarditis in 1 per cent of 132 normal control animals.

Results

Acute Experiments

Table 1 shows that the production of cardiac damage in young rabbits is readily accomplished. Some significant alteration in the heart was noted in 49 of the 58 animals challenged, in contrast to no involvement in the untreated control group of 21 animals. The cardiac-damaging factor did not seem to be limited to agents of streptococcal origin, but serum proteins from both man and cow seemed to possess this activity. Note that these human and bovine proteins were conjugated with the fluorescent dye lissamine rhodamine. The distribution of these materials, including the conjugated streptococcal sonicate, will be the subject of a future publication.

During the course of the experimentation it was noted that different rabbits, as evidenced by the tissue reaction, reacted differently to a given agent even when the dosages administered were equal. For purposes of description, the type of tissue reaction in the heart has been classified on a histological basis. The results have been classified into four main types, undoubtedly with overlapping. This classification is based on the type and distribution of the lesions and in no way is to be construed as related to the degree of biological activity of the agent or to the dosage employed.[20] The latter point is well demonstrated by the finding that all of the rabbits listed in table 2 showed the same type of reaction, type 4, despite the fact that various dosages of the streptococcal sonicate were employed. The following types were found:

Type 1. Small isolated focal collections of mononuclear cells and a few lymphocytes scattered throughout the myocardial connective tissue. There was little or no evidence of connective tissue or muscle cell damage. These

TABLE 1
INCIDENCE OF CARDIAC LESIONS IN RABBITS

Material	Dose*	Total No. rabbits	No. with carditis					No. without carditis
			Total	Type 1	2	3	4	
Type I strept. sonicate	1 inject.	14	11	2	1	1	7	3
	2 inject.	3	3	1	—	1	1	—
Rhodamine-conjugated type I strept. sonicate	1 inject.	6	6	2	3	1	—	—
	2 inject.	8	8	2	—	2	4	—
Type VI strept. sonicate	1 inject.	7	2	—	—	—	2	5
	2 inject.	10	9	4	4	1	—	1
Type XII strept. broth filtrate	5 cc.	6	6	2	2	1	1	—
Rhodamine-conjugated bovine serum albumin	40 mg./kg.	2	2	—	2	—	—	—
Rhodamine-conjugated human serum protein	60 mg./kg.	2	2	—	2	—	—	—
	Totals	58	49	13	14	7	15	9
Untreated controls		21	—	—	—	—	—	21

* Administered intravenously unless stated otherwise in the text.

TABLE 2
ACUTE CARDIAC EFFECTS RELATED TO SIZE OF DOSE
(Type I streptococcus sonicate in 1 injection)

Dose I.V.	Total No. rabbits	No. with carditis*	No. without carditis
1 mg./kg.	2	1	1
2 mg./kg.	3	2	1
3 mg./kg.	3	3	—
4 mg./kg.	2	1	1†
Total	10	7	3

* All rabbits showed type 4, or generalized acute interstitial myocarditis damage and lesions were limited to the "right" heart.

† Rabbit died spontaneously within 18 hours after injection and was autopsied immediately.

aggregates of mononuclear cells were not numerous, and seemed to have no definite relationship to blood vessels (FIGURE 1).

Type 2. Larger focal collections of mononuclear cells within the myocardium, with minimal evidence of muscle and connective tissue damage. Not infrequently, these cellular collections were found in the perivascular connective

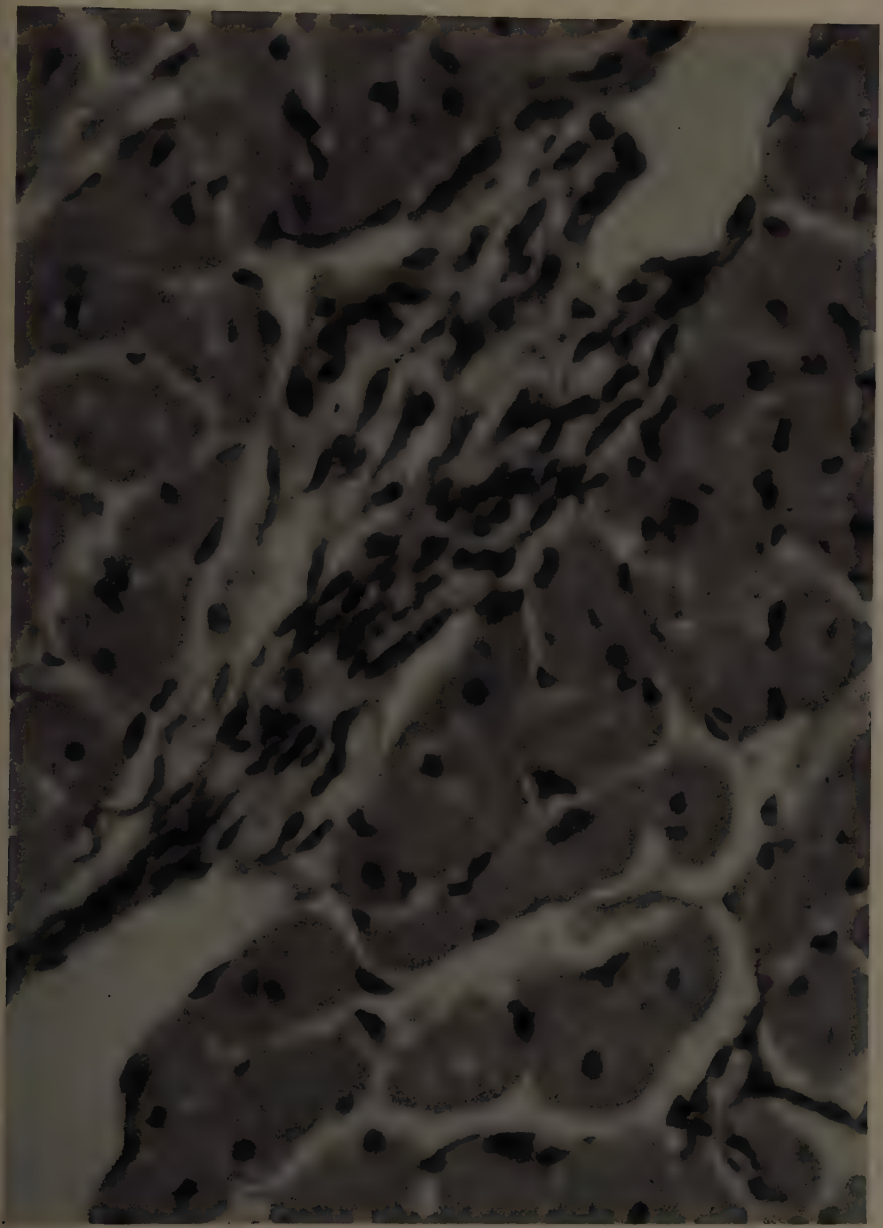

FIGURE 1. Rabbit 50. Two intravenous injections of type VI streptococcal sonicate. Note the focal collection of mononuclear cells and occasional Anitschkow histiocytes in the interstitial tissue of the left ventricular myocardium. There is a lack of fibrinoid and an absence of characteristic Aschoff cells. Hematoxylin and eosin. ×125.

tissue of the coronary arteries, and the Anitschkow-type histiocyte commonly was seen. In the majority of these hearts, a mild degree of arteritis was noted, as evidenced by intense infiltration of the adventitia by typical Anitschkow cells (FIGURE 2). Fibrinoid alteration of the affected vessels was noted rarely in our animals. (The term Anitschkow cells refers only to those mononuclear cells with large oval nuclei and rather vaguely defined cytoplasm. On longitudinal section, a heavy chromatin mass with distinctive fibrillar network extending to the nuclear membrane is seen, thus giving rise to its unmistakable appearance. On cross section, the heavy chromatin mass in the center of the nucleus with its fibrillar network is responsible for its "owl-eyed" appearance.[32])

Type 3. This type includes not only the findings listed under type 2, but also evidence of focal subendocardial collections of these mononuclear cells, frequently within the valves (FIGURE 3*a* and *b*). More usually, however, the valvular involvement was manifested as hemorrhage into the valve substance itself. There seemed to be no specificity of valvular involvement (TABLE 3).

Type 4. There is a widespread, often confluent, patchy interstitial myocarditis with connective tissue and muscle fiber damage. The inflammatory cells found scattered throughout this process were predominantly polymorphonuclear leukocytes and lymphocytes. Arteritis, as described above, was found rather infrequently, although 4 of the animals exhibiting these changes also had gross hemorrhages into the valves (FIGURE 4).

As stated above, the type of damage did not correlate with either the agent or the dosage employed (TABLES 1 and 2). The type of damage seemed completely unpredictable and, although we tried to maintain the same conditions throughout, it was not possible to carry out experimentation throughout the year without variations. However, it may be seen readily from the results of the untreated rabbits listed in TABLE 1 that there was no evidence of "spontaneous" carditis.

Regardless of the type of involvement produced by the agent, all affected animals except 3 manifested some form of myocarditis, as revealed by TABLE 3. The majority of these animals also exhibited some form of arteritis, characterized by a generalized thickening of the vessels involved, usually the larger coronary arteries, and a diffuse infiltration of the media and adventitia with large mononuclear cells and typical Anitschkow cells (FIGURE 2). The polyarteritis-type lesions seen by Rich and Gregory[4] in their rabbits given foreign serum proteins were not seen in our animals.

The valvular involvement seemed to be of a nonspecific, random nature, being noted in 10 of the 49 affected animals. There was no preferential localization of valvular damage, the mitral, aortic, and tricuspid valves being equally involved. Grossly at autopsy, valvular hemorrhage was seen, but no significant nodularity or beading was evident. Microscopically, the most common finding was that of hemorrhage in the valve substance, although 4 animals showed a focally increased collection of large mononuclear and Anitschkow cells, as shown in FIGURE 3*b*. There was very little alteration of the valvular collagen fibers.

The right side, or pulmonary circuit, of the heart showed the same degree of involvement as the left side, or systemic circuit. This is contrary to the

FIGURE 2. Rabbit 4. One intravenous injection of type VI streptococcal sonicate. A larger coronary artery branch in the left ventricular myocardium, showing rather marked hyperplastic changes. The increased number of Anitschkow histiocytes seems to be "streaming out" into the perivascular connective tissue. Note the lack of fibrinoid involvement of the wall of the vessel. Hematoxylin and eosin. ×200.

FIGURE 3. (*a*) Rabbit 1. One intravenous injection of type I streptococcal sonicate. There is a focal subendocardial collection of round cells in the left ventricle (type II change). Hematoxylin and eosin. ×150.

observations made by Herbertson[17] and others[21] of almost exclusive involvement of the right side. However, in our last series of rabbits, during the production of lesions by a single injection of the type I streptococcal sonicate in varying doses, only an acute interstitial type of myocarditis was noted, which was limited to the right side of the heart. Herbertson believes that the exclusively right-sided lesion is due to myocardial ischemia secondary to the distention and dilation of the right chambers.

FIGURE 3. (*b*) Rabbit 42. Two intravenous injections of type I streptococcal sonicate. Focal collections of mononuclear cells in the mitral valve near the valve sulcus. Mild connective tissue swelling. Hematoxylin and eosin. ×50.

Long-Term Experiment

The results shown in TABLE 4 are especially interesting in view of the fact that the lesions were all of a mild nature. There were small focal collections of lymphocytes and mononuclear cells similar to the type I category, but the mild arteritis described above was invariably seen. However, there was widespread fibrosis throughout the myocardium, although the remaining muscle cells appeared normal. (FIGURE 5).

It seems that these lesions represent a reparative process with resultant fibrosis. Eventually, there is no trace of the original carditis except for increased patchy scarring throughout the myocardium.

Histochemical Studies

TABLE 5 summarizes the results obtained when the experimental carditis lesions were compared with the mature Aschoff body. The changes in the acid

FIGURE 4. Rabbit 31. One intravenous injection of type XII streptococcal broth filtrate. There is widespread acute interstitial myocarditis in the left ventricular myocardium. Note the relative absence of inflammation around the intramyocardial coronary vessel. Hematoxylin and eosin. ×100.

mucopolysaccharides and collagen fibers in the experimental lesions appeared to be related to the intensity of the cellular infiltrate and its relationship to the perivascular stroma. However, the most severely involved areas did not show

TABLE 3
SITES OF CARDIAC INVOLVEMENT

Material	Dose	Total No. rabbits	No. without carditis	No. with carditis	Myocardium	Blood vessel	Valves	Side of heart*	
								Right	Left
Type I strept. sonicate	1 inject.	14	3	11	10	4	2 {1 mitral, 1 tricuspid}	8	4
	2 inject.	3	—	3	3	2	1	3	3
Rhodamine-conjugated	1 inject.	6	—	6	5	5	1	4	6
Type I strept. sonicate	2 inject.	8	—	8	7	7	4 {2 mitral, 1 aortic, 1 tricuspid}	7	6
Type VI strept. sonicate	1 inject.	7	5	2	2	2	1 tricuspid	2	2
	2 inject.	10	1	9	9	9	1 mitral	8	9
Type XII strept. broth filtrate	5 cc.	6	—	6	6	3	—	4	6
Rhodamine-conjugated bovine serum albumin	40 mg./kg.	2	—	2	2	2	—	1	2
Rhodamine-conjugated human serum protein	60 mg./kg.	2	—	2	2	—	—	1	1
	Total	58	9	49	46	34	10	38	39

* Side of heart refers to either pulmonary or systemic circuit. In sections of the interventricular septum an attempt was made to differentiate the "side" of involvement.

TABLE 4
LONG-TERM CARDIAC EFFECTS OF STREPTOCOCCAL TYPE I SONICATE

Material	Total	No. without carditis	No. with carditis	Myocardium*	Blood vessel	Valve
Freund's adjuvant with *M. butyricum*	5	5	—	—	—	—
Freund's adjuvant with *M. butyricum* and type I streptococcus sonicate	10	2	8	8	7	—

* Myocarditis was all of type I and marked fibrosis usually was noted.

the collagen fiber alterations and the increased number of mucopolysaccharides characteristically associated with the Aschoff body. The reactivity of the cytoplasm of the Anitschkow and Aschoff cells in rheumatic carditis is different from that of the Anitschkow cells observed in the normal rabbits. Gamma globulin localization in Aschoff bodies by the use of fluorescein-labeled rabbit

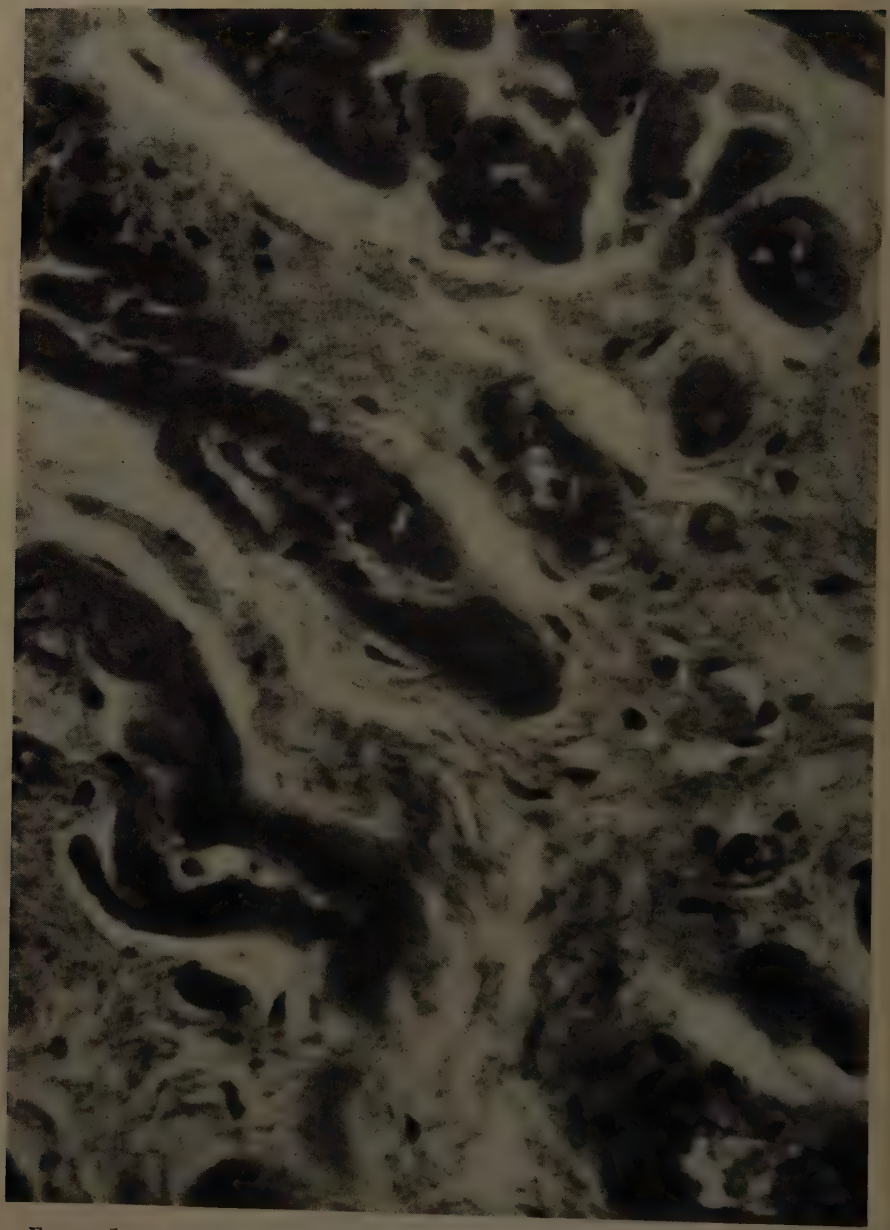

FIGURE 5. Rabbit 70. Long-term experiment, streptococcal type I sonicate. Sacrifice 120 days after initial injection. Note the myocardial fibrosis and loss of myofibers. There is a striking lack of inflammatory cells. Hematoxylin and eosin. ×125.

antihuman gamma globulin showed variable results. Presumably, this represents serum gamma globulin in an area of inflammation. The possibility that the gamma globulin noted in relation to the Aschoff body is antibody cannot be excluded.[33]

Discussion

The production of carditis in New Zealand rabbits may be accomplished with a variety of unrelated materials. In the present study, a significant number of animals showed lesions suggestive of the constellation of changes constituting the Aschoff body. The rabbit lesions were focal, Anitschkow cells were multiplied, and orientation was frequently perivascular and subendocardial. These are alterations that may be observed in the development of the pathognomonic Aschoff body, but the critical features of the Aschoff body were not observed under the conditions of the present experiment. Murphy and Swift[34] claimed to have successfully duplicated the Aschoff body in their experimental rabbits by successive intracutaneous injections of differing types of living group A streptococci. Since a small percentage of rabbits, after 3 to 20 months and many repeated cutaneous infections, developed the characteristic lesions, they concluded that some host factor played a part in the pathogenesis of these tissue alterations. However, most of the lesions in the illustrations accompanying the text seem to show primary muscle cell involvement closely simulating the myogenic-type giant cell not uncommonly seen in nonrheumatic cardiac disorders.[35]

Kirschner and Howie[36] reported that they had produced comparable cardiac lesions in 7 of 12 rabbits similarly injected. Recently, Norlin,[37] under similar circumstances, also produced cardiac lesions in 7 of 32 test animals. However, Robinson[38] failed to reproduce the results of Murphy and Swift,[34] although he was able to produce widespread cardiac damage. L. E. Glynn and T. N. Harris (personal communications) have also attempted to confirm the results claimed by Murphy and Swift[34] with little success.

It is quite obvious that the crux of the controversy centers on the criteria utilized for the identification of the Aschoff body. The application of newer techniques to this problem may help to resolve the current dilemma. Thus, TABLE 5 shows the distinct differences between the experimental and human processes.

The present study is part of a long series of investigations designed to test experimental systems that might result in reproduction of the rheumatic state. Schwab *et al.*[40] claim that the toxic component of the streptococcal sonicate is a complex of group-specific C polysaccharide and protein. However, W. J. Cromartie (personal communication) thinks that the cardiac changes observed in this study probably were due to "one or more of a great variety of substances contained in the crude extract and not to the rather large macromolecules of C-polysaccharide." This is difficult to assess, since rhodamine-labeled serum proteins and crude streptococcal sonicate rapidly leave the circulation and enter the cardiac valvular and myocardial connective tissues.[40] Streptococcal sonicate is an extremely potent biological material; Stetson[41] noted the similarity of streptococcal lysates (which may be considered similar

to our sonicate[42]) to Gram-negative bacterial endotoxins in their ability to produce fever, leukopenia, and peripheral vasomotor reactions, as well as the local and generalized Schwartzman phenomena.

In comparing the experimentally produced lesions in this study with those of other investigators, it seems fair to state that they bear a striking similarity. There is an apparent closer resemblance of these experimental lesions to those seen in acute streptococcal myocarditis.[43,44] We were unsuccessful in producing a further evolution of the cardiac changes by first sensitizing the rabbit to the crude streptococcal sonicate and then repeatedly injecting the same sonicate intravenously. A long-term experiment is currently in progress, whereby repeated intradermal injections of the streptococcal sonicate with and without Freund's adjuvant are being administered to rabbits. After 6 months of experimentation the rabbits continue to be healthy.

TABLE 5
HISTOCHEMICAL STUDIES OF EXPERIMENTAL CARDITIS AND RHEUMATIC CARDITIS (ASCHOFF BODY)*

	Rheumatic	Experimental
Collagen fiber changes	++ to +++	± to +
Acid mucopolysaccharides	increased	variable increased
Anitschkow cells	increased	increased
RNA in cytoplasm	+++	++
Reactive NH_2 groups	+++	++
Nonspecific esterase	+++	—
Acid phosphatase	+++	—
Gamma globulin localization	+ to ++	+ to ++
Fibrinoid	± to +	—
Myogenic giant cells	—	+ to ++
Aschoff cells	+++	—

* No change, —; variable change, ±; intensity of reaction, + to +++.

Finally, the production of these myocardial lesions in such diverse ways and with such a wide variety of agents throws doubt upon the specificity of this reaction in the rabbit heart. Utilizing meningococcal endotoxin, *E. coli* lipopolysaccharide, streptococcal proteinase, and horse serum in acute experiments with rabbits, similar observations have been made. The mechanisms responsible for the strikingly comparable tissue changes are largely unknown. However, there is clearly a limited responsiveness of cardiac tissues to acute stress. It indicates that a great deal more caution must be taken in evaluating experimentally produced cardiac lesions in this host, especially in comparing the changes with human rheumatic fever.

SUMMARY

Several biologically active agents administered systemically to stock New Zealand rabbits produce carditis. Extracts prepared from sonically disrupted group A streptococci belong to this group. The acute effects of such sonicates on cardiac tissues are described. The morphologic alterations noted differ sharply from human rheumatic fever.

Acknowledgment

Sincere appreciation is expressed to William Cromartie and Russell Weiser for their advice and generous help in these studies. Preparation of the streptococcal sonicates was facilitated by the technical assistance of Edwin Boatman.

References

1. McCarty, M. 1956. Nature of rheumatic fever. Circulation. **14:** 1138.
2. Rantz, L. A. 1955. The streptococcal etiology of rheumatic fever. Med. Clin. N. Am. **39:** 339.
3. Long, D. A. 1954. The pathogenesis of rheumatic fever. Lancet. **266:** 529.
4. Rich, A. R. & J. E. Gregory. 1943. Experimental evidence that lesions with the basic characteristics of rheumatic carditis can result from anaphylactic hypersensitivity. Bull. Johns Hopkins Hosp. **73:** 239.
5. Klinge, F. 1933. Der Rheumatismus. Bergmann. Munich, Germany.
6. Ehrich, W. E., J. Seifter & C. J. Forman. 1948. Experimental serum disease: a pathogenetic study. J. Exptl. Med. **89:** 23.
7. Wissler, R. W., K. Smull & J. Lesh. 1949. The effects of various horse serum fractions in producing cardiovascular and renal lesions in rabbits. J. Exptl. Med. **90:** 577.
8. More, R. H., D. E. Waugh & S. D. Kobernick. 1949. Cardiac lesions produced in rabbits by massive injections of bovine serum gamma globulin. J. Exptl. Med., **89:** 555.
9. Cole, R. I. 1904. Experimental streptococcus arthritis in relation to the etiology of acute articular rheumatism. J. Infectious Diseases. **1:** 74.
10. Magrassi, F. 1933. Experimental infectious rheumatism and streptococcal focal infection. Acta Rheumatol. **5:** 2.
11. Robinson, J. J. 1951. Attempts to produce rheumatic carditis in laboratory animals by means of streptococcic injury. A.M.A. Arch. Pathol. **51:** 602.
12. Kellner, A. & R. Robertson. 1954. Myocardial Necrosis Produced in Animals by Means of Crystalline Streptococcal Proteinase. J. Exptl. Med. **99:** 495.
13. Kellner, A. 1954. Selective necrosis of cardiac and skeletal muscles induced by means of proteolytic enzyme solutions given intravenously. J. Exptl. Med. **99:** 387.
14. Miller, C. P. 1924. Attempts to transmit rheumatic fever to rabbits and guinea pigs. J. Exptl. Med. **40:** 525.
15. Swift, H. F. 1936. The nature of rheumatic fever. J. Lab. Clin. Med. **21:** 551.
16. Cavelti, P. A. 1947. Studies on the pathogenesis of rheumatic fever. A.M.A. Arch. Pathol. **44:** 1.
17. Herbertson, B. M. 1956. Patchy myocardial necrosis in rabbits after shock doses of histamine and peptone. J. Pathol. Bacteriol. **72:** 137.
18. Selye, H. 1958. The Chemical Prevention of Cardiac Necrosis. Ronald. New York, N. Y.
19. Wagner, B. M. 1957. Relationships of the Aschoff body to experimental carditis. Federation Proc. **16:** 376.
20. Wagner, B. M., P. Vanace & J. McGrath. 1958. Hypersensitivity myocarditis in rabbits and swine. Federation Proc. **17:** 462.
21. Thomas, L., F. W. Denny & J. Floyd. 1955. Studies on the generalized Schwartzman reaction. III. Lesions of the myocardium and coronary arteries accompanying the reaction in rabbits prepared by infection with group A streptococci. J. Exptl. Med. **97:** 751.
22. Jones, R. S. & Y. Carter. 1954. A study of the pathogenesis of rheumatic-like lesions in the guinea pigs A.M.A. Arch. Pathol. **58:** 613.
23. Parker, B. M., W. A. Thomas, J. R. Smith & R. C. Ahlvin. 1957. Myocarditis and valvulitis in dogs subsequent to cardiovascular stress. A.M.A. Arch. Pathol. **64:** 522.
24. Aschoff, L. 1904. Zur Myocarditis Frage. Verhand. I. D. Deut. Pathol. Ges. **8:** 46.
25. Saphir, O. 1959. The Aschoff nodule. Am. J. Clin. Pathol. **31:** 534.
26. Bagenstoss, A. H. 1953. Rheumatic disease of the heart. *In* The Pathology of the Heart, S. E. Gould, Ed. Thomas. Springfield, Ill.
27. Wagner, B. M. 1960. Studies in Rheumatic Fever. III. The Aschoff Body. Ann. N. Y. Acad. Sci. **86:** 992–1008.
28. Schwab, J. H. & W. J. Cromartie. 1957. Studies on a toxic cellular component of group A streptococci. J. Bacteriol. **74:** 673.
29. Schwab, J. H. 1956. An intracellular hemolysin of group A streptococci. I. Influence of sonic energy and *p*H on hemolytic potency. J. Bacteriol. **71:** 94.

30. Chadwick, C. S., M. G. McEntegart & R. C. Nairn. 1958. Fluorescent protein tracers. A trial of new fluorochromes and the development of an alternative to fluorescein. Immunology. **1:** 315.
31. Miller, C. P. 1924. Spontaneous interstitial myocarditis in rabbits. J. Exptl. Med. **40:** 543.
32. Loewe, L. & S. E. Lenke. 1940. Cardiac lesions resembling Aschoff bodies in rabbits. J. Exptl. Med. **71:** 89.
33. Ehrlich, J. C. & B. Lapan. 1939. The Anitschkow "myocyte." A.M.A. Arch. Pathol. **361:** 370.
34. Wagner, B. M. 1957. Hypersensitivity: The Role of the Connective Tissue in Analytical Pathology. R. Mellors, Ed. McGraw-Hill. New York, N. Y.
35. Murphy, G. E. & H. F. Swift. 1949. Induction of cardiac lesions, closely resembling those of rheumatic fever, in rabbits following repeated skin infections with group A streptococci. J. Exptl. Med. **89:** 687.
36. Reubner, B. 1954. The relationship between muscle damage and the Aschoff cell in rheumatic carditis. J. Pathol. Bacteriol. **68:** 101.
37. Kirschner, L. & J. B. Howie. 1952. Rheumatic-like lesions in the heart of rabbits experimentally induced by repeated inoculations with hemolytic streptococcus. J. Pathol. Bacteriol. **64:** 367.
38. Norlin, G. 1959. Experimental heart granulomatosis in rabbits and its relation to rheumatic carditis. Acta Rheumatism Scand. **5:** 85.
39. Robinson, J. J. 1954. Failure to produce rheumatic fever in rabbits by prolonged and intensive streptococcal infections. A.M.A. Arch. Pathol. **57:** 516.
40. Schwab, J. H., W. J. Cromartie & B. S. Roberson. 1959. Identification of a toxic cellular component of group A streptococci as a complex of group specific C polysaccharide and a protein. J. Exptl. Med. **109:** 43.
41. Char, D., E. Boatman & B. M. Wagner. To be published.
42. Stetson, C. A. 1956. The endotoxic properties of lysates of group A hemolytic streptococci. J. Exptl. Med. **104:** 921.
43. Hess, E. L. & H. D. Slade. 1955. An electrophoretic examination of cell free extracts from various serological types of group A hemolytic streptococcus. Biochim. et Biophys. Acta. **16:** 346.
44. Mallory, G. K. & C. S. Keefer. 1941. Tissue reactions in fatal cases of streptococcus hemolyticus infection. A.M.A. Arch. Pathol. **32:** 334.
45. Watson, R. F., S. Rothbard & H. F. Swift. 1945. The relationship of postscarlatinal arthritis and carditis to rheumatic fever. J. Am. Med. Assoc. **128:** 1145.

IMMUNOPATHOLOGY OF HYPERSENSITIVITY*

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The use of such recently developed techniques as quantitative immunochemistry, electron microscopy, and immunohistochemical methods accounts in part for the renewed interest in a group of diseases collectively named connective tissue diseases. It is the purpose of this article to review some of the results and interpretations of our studies of both human and experimental conditions in this group of diseases, in which these techniques (chiefly the fluorescent antibody method of Coons *et al.*[1,2]) were used. It would be repetitious to review the numerous papers stressing the common aspects of the so-called connective tissue diseases, the reasons for grouping them together, and their possible pathogenetic mechanisms such as those based on hypersensitivity, especially. Acknowledgment is made of studies similar to those presented here.[3]

If one accepts the hypothesis that a lesion of hypersensitivity may involve an antigen-antibody reaction, the fluorescent antibody technique can test for the presence of such reactants at the lesion. It is possible to take this direct approach to certain experimental conditions such as "serum sickness" and related states, but in the case of human diseases of suspected hypersensitivity origin little or no information is available concerning possible antigen or, thereby, antibody, making this approach quite difficult. One approach that may be used, however, is that of testing human lesions for the presence of homologous gamma globulin on the assumption that this protein will indicate antibody. With the fluorescent antibody technique, still a third approach has been used, that of determining the presence of tissue-localizing antibody in the serum of a given case, on the assumption that, if any is found, it may be instrumental in the production of a pathological change. It should be mentioned that, although many positive and interesting results have been obtained by these methods, one of the greatest difficulties has been the interpretation of results, that is, of assigning to a given immunological reactant a role in the pathogenesis of a lesion or disease.

The studies to be presented are grouped under the following headings: (1) diseases in which a direct demonstration of antigen, antibody, or both has been made at the lesion sites, (2) diseases in which a significant concentration of a given plasma protein (gamma globulin or either fibrinogen or fibrin) has been found, and (3) diseases in which the serum contains tissue-localizing "antibodies."

Diseases with Direct Demonstration of Antigen or Antibody or Both at Lesion Sites

Studies in this group will include experimental serum sickness, experimental chronic glomerulonephritis, the Arthus reaction, and anaphylaxis.

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Reports from our laboratory[4,5] have shown that, in serum sickness produced by a single large intravenous dose of a purified antigen (bovine serum albumin) in the rabbit, the initiation and development of the morphologic manifestations (1) parallel the increase in rate of antibody production, (2) occur while antigen-antibody complexes shown to be capable of inducing inflammation are present in the circulation, and (3) are accompanied by a simultaneous localization of antigen and, probably, of antibody in the sites of the lesions (coronary arteritis and acute glomerulitis). It also has been shown that there was no fixation of antigen in those tissues predisposed to the development of lesions prior to the formation of antibody. The regression of the lesions of serum sickness (1) followed the disappearance of readily detectable circulating antigen-antibody complexes, (2) paralleled a decline in rate of antibody production, and (3) were accompanied by the disappearance of antigen from the lesions. With these results, a mechanism by which the interaction of antigen and antibody may cause the development of the lesions of serum sickness was suggested. The events might run as follows:

(1) As antibody begins to appear it combines with antigen in antigen excess forming soluble complexes that increase in amount with the increase in antibody synthesis until all antigen is eliminated.

(2) These circulating complexes presumably are capable of initiating inflammatory responses in the host.

(3) By means not yet known, these complexes are localized in the inflammatory lesions initiated by them (arteries and glomeruli), the development of the lesions and the localization and concentration of complexes in such lesions progressing simultaneously and perhaps enhancing each other.

(4) The antigen-antibody complexes are eliminated from the circulation, the lesions of serum sickness promptly disappear, and in the process the antigen and antibody in them are catabolized.

As previously mentioned, the lesions in serum sickness found by the procedure outlined are reversible but, although the glomerulitis found in such instances has been likened to that found in human glomerulonephritis, a chronic irreversible type of glomerular change is not achieved by this procedure. An experimental glomerulonephritis quite similar to the irreversible chronic human glomerulonephritis has been produced in some rabbits by daily intravenous injections of horse serum[6] or, more recently,[7] by daily intravenous injections of a purified antigen (bovine serum albumin), for a period of 4 weeks and more, the doses ranging from 10 to 100 mg. In these animals a typical membranous or chronic proliferative glomerulonephritis, or both, indistinguishable from the human counterpart, were produced. Using the fluorescent antibody technique appreciable amounts of specific antigen and, presumably, of antibody were found in the altered glomeruli which, by comparative electron microscopy, were found to be localized in the basement membranes.[8] No lesions in other viscera were found. The rabbits receiving daily doses of antigen fell into the three immunological groups outlined above: (1) a group in which great amounts of circulating antibody were found, (2) a group in which no circulating antibody was detected, and (3) a group in which the animals barely make enough antibody to combine with all the circulating antigen present. It is in this last

group that the characteristic chronic lesions described appear. A possibility currently under investigation is that the development of these renal lesions is dependent on the presence in the circulation of antigen-antibody complexes in a certain antigen-antibody ratio (soluble complexes).

Studies in our laboratory[9,10] have shown that specific antigen and, presumably, antibody are found in affected vessel walls manifesting both the active or classic Arthus reaction and the reverse (passive) Arthus reaction. It has been found also that the polymorphonuclear leukocyte played an essential role not only in producing the vasculitis but also in eliminating the antigen from the damaged vessel, probably by means of proteolytic catabolism at the inflammatory site. A temporal relationship between the disappearance of antigen from the damaged vessels and a decrease in the inflammatory reaction was found. The earliest localization of antigen in the Arthus vasculitis was found to be beneath the endothelium of small vessels. In further studies of the Arthus reaction tests were made of the *in vivo* activity of soluble antigen-antibody complexes intradermally injected in rabbits. The results showed that such antigen-antibody complexes did produce inflammatory changes, and provided support to the suggestion that complexes were responsible for the lesions of experimental serum sickness.

Anaphylaxis in the rabbit, as reported previously,[11] shows eosinophilic precipitates in the lumens of vessels throughout the body, such precipitates being composed of specific antigen and, presumably, antibody when tested by immunohistochemical techniques.[7,12]

Results obtained with an experimental approach such as that described above seem to give firm support to the inference that certain inflammatory or degenerative lesions or both result from the interaction of antigen and antibody. The lesions of serum sickness and related experimental states have been likened to many of the human lesions in the connective tissue disease group because of the sometimes striking morphologic similarities. If one admits a certain limited capacity of connective tissue to react in different ways to any given stimulus, one should be cautious in supposing an absolute correlation between experimental and human conditions. With the information available it is only safe to suggest a possible pathogenetic similarity between these different disease states. More convincing evidence of an immunologic mechanism in some of the human diseases should rest on the direct identification of immunological reactants at the lesion sites.

Diseases with Specific Concentration of Given Plasma Protein (Gamma Globulin or Either Fibrinogen or Fibrin)

As mentioned earlier in this presentation, a direct demonstration that an immunological mechanism plays an essential role in human connective tissue diseases still is lacking. Since in almost all cases a possible offending antigen is not well characterized or is not known, a specific identification of antigen, antibody, or both in the lesions is not possible. Nevertheless, it may be assumed that an antigen-antibody reaction in the tissues could result in local increased concentrations of the host's gamma globulin, if such protein represents antibody. A second immunohistochemical approach was undertaken in

the hope, first, that a relatively simple analysis of the plasma protein composition of the lesions of several of the connective tissue diseases would show a preferential concentration of gamma globulin at the lesion sites and, second, that similarities or differences among the various entities would be determined. The fluorescent antibody technique was employed in determining the relative concentrations of gamma globulin, fibrinogen or fibrin, and albumin in the lesions of a number of human diseases.[13] On the basis of the observations made, the conditions could be grouped as those with lesions showing a preferential concentration of homologous gamma globulin and those with lesions showing a preferential concentration of fibrinogen, fibrin, or both, as follows:

(1) Diseases with preferential concentration of gamma globulin: (a) experimental hypersensitivity of the "immediate type," including serum sickness, Arthus reaction, anaphylaxis, and chronic glomerulonephritis; (b) systemic lupus erythematosus; (c) rheumatic fever; (d) rheumatoid arthritis; (e) polyarteritis nodosa;[14] (f) human glomerulonephritis;[14] and (g) human and experimental amyloidosis.

(2) Diseases with preferential concentration of fibrinogen, fibrin, or both: (a) generalized and local Shwartzman reaction; (b) thrombotic thrombocytopenic purpura; (c) bilateral cortical necrosis in complications of pregnancy; (d) renal lesions in systemic scleroderma;[15] and (e) malignant hypertension.[15]

Our findings in human and experimental amyloid merit brief comment.[16] In experimental amyloidosis induced by repeated casein injections that elicit prolonged antibody response and in that induced by repeated ribonucleic acid injections that cause no detectable antibody response the amyloid deposits are morphologically similar. In both experimental and human conditions the amyloid contains significant concentrations of the host's gamma globulin. In casein-induced amyloid, antibody and, presumably, antigen have been identified in the amyloid deposits. However, whether these specific reactants are the result of an immunological reaction important in the pathogenesis of amyloidosis or whether they represent a nonimmunological deposition of the host's gamma globulin, of which some is specific antibody and thus capable of combining with injected antigen, remains to be determined. Since a similar deposition of amyloid containing gamma globulin can occur in the absence of a detectable antibody response, it would appear that immunological mechanisms are not essential to amyloid formation.[17] The immunochemical test results of concentrations of gamma globulin in amyloid are in agreement with the reaction of I^{131}-labeled anti-gamma globulin and amyloid and are also consistent with the short half life of circulating gamma globulin in this disease.

While these observations serve to divide the diseases studied into two groups according to the plasma protein composition of the lesions, they do little to explain the pathogenic mechanisms involved. Certainly, the localized antigen-antibody reactions that occur in experimental serum sickness and related states are associated with a localization of gamma globulin in the lesions. However, the observations of experimental amyloidosis indicate that identical lesions with similar concentrations of gamma globulin may be induced with two different procedures, one of which is associated with an immunological phenomenon and one of which is not. A second possible explanation of gamma globu-

lin concentration in lesions is that primary connective tissue changes increase the affinity of that tissue for gamma globulin. There is normally a slight affinity of gamma globulin for some tissue constituents, and this may be magnified in such situations. A third possible explanation is that a metabolic alteration leading to hypergammaglobulinemia frequently found in some of these diseases predisposes to the nonimmunological deposition of gamma globulin in normal or in abnormal tissues. In our present state of ignorance regarding the so-called connective tissue diseases, such observations indicate only the differences and, perhaps less surely, the similarities between some of the entities and emphasize the fact that we are far from being able to apply a unifying pathogenetic concept to the study of this group of diseases.

Diseases with Serum Containing Tissue-Localizing "Antibody"

In the past few years there has been a renewed interest in autoimmunity as a possible cause of a variety of diseases, chiefly those called connective tissue diseases. The demonstration of autoimmunization in both humans and experimental animals has disproved the theory of "horror autotoxicus," but still leaves questions unanswered: (1) In how many diseases do autoimmune mechanisms operate? (2) What role do they play in those diseases? The precise nature of these mechanisms in most instances is still not understood. This lack of understanding and the inability to transfer passively an etiological agent in most diseases associated with autoimmunity make it extremely difficult to evaluate the role of any autoimmune reaction in the pathogenesis of the diseases.[18] For example, serum factors possessing most or all of the characteristics of autoantibodies with tissue-localizing properties have been demonstrated in human and experimental thyroiditis, systemic lupus erythematosus, rheumatoid arthritis, and others. In some cases a strong suggestion has been made that circulating autoantibodies are responsible for the development of the lesions, thus proving their autoimmune pathogenesis. However, whether these autoimmune responses mediate the essential pathological processes in most of the diseases where they are found or whether they are merely secondary phenomena in the course of a host response remains to be established.

Recently we have had the opportunity of studying two unusual patients showing identical clinicomorphologic manifestations that warranted in each case the eponymic diagnoses of Hashimoto's thyroiditis, Sjögren's disease, and Waldenström's macroglobulinemic purpura. The spectrum of diseases in these two patients implied a single disease process with various clinical manifestations (J. J. Vazquez and W. N. Jensen, unpublished observations). To evaluate a possible role of autoimmunity in the pathogenesis of the disease in these patients and, if such a role existed, to determine whether a degree of tissue specificity was present, we labeled with fluorescein the globulin fractions of each of the two patients' sera, for immunohistochemical stains in sections of these patients' tissues and of normal tissues. One patient's labeled serum caused staining of *nuclear* elements of tissues from the two patients and from the normal persons (FIGURE 1), similar to the nuclear staining with labeled serum in lupus erythematosus. Labeled serum from the other patient stained only the thyroid colloid and cytoplasm of thyroid follicular cells from both

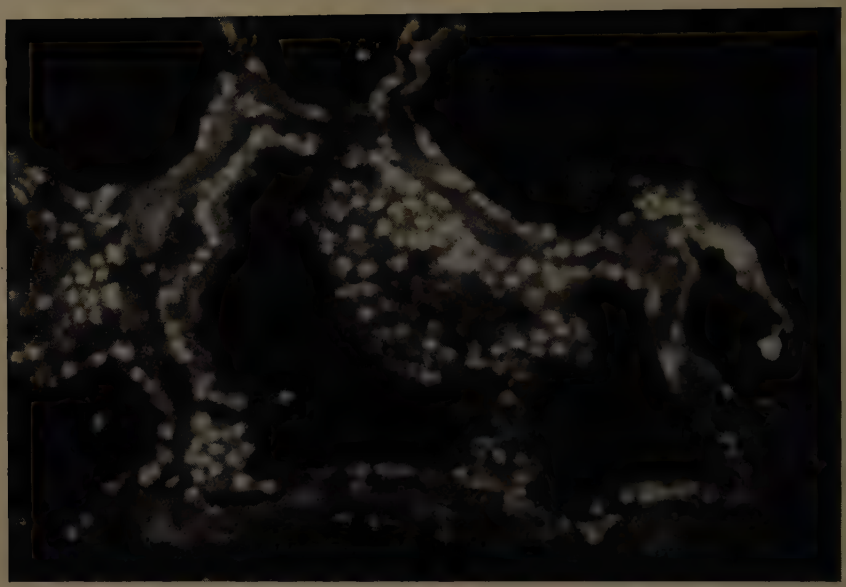

FIGURE 1. Fluorescent photomicrograph of a frozen section of normal human thyroid stained with fluorescein-conjugated globulin fraction of serum of one of two patients showing Hashimoto's disease, Sjögren's disease, and macroglobulinemia. Note the bright fluorescence of nuclei of cells lining the follicles, indicating a factor in the conjugate with properties of tissue localization specific to nucleus. Colloid material does not appear in this section, probably because of washing off in the process of staining; however, in other sections the colloid material was present and it lacked specific fluorescence. No cytoplasmic fluorescence was observed in this case.

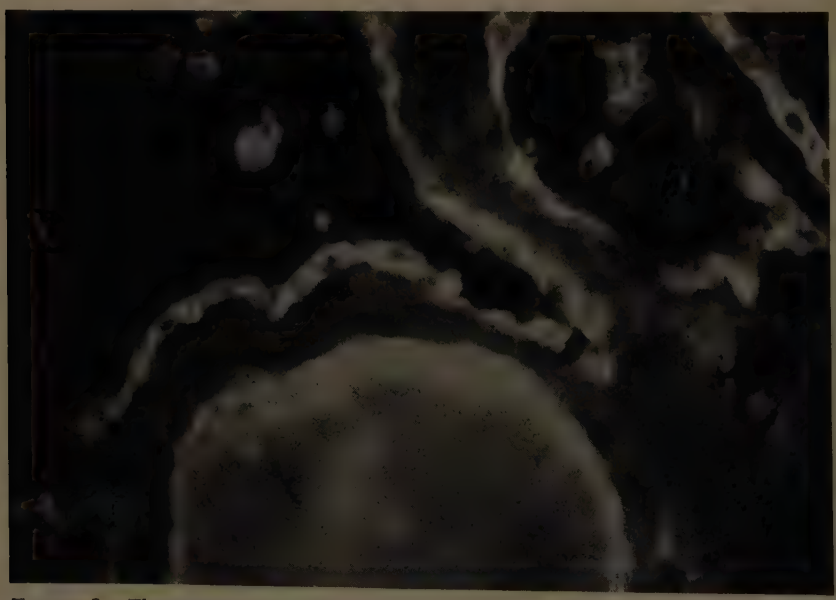

FIGURE 2. Fluorescent photomicrograph of normal human thyroid stained with fluorescent conjugate of the globulin fraction of the other patient, showing same clinicomorphologic changes as patient in FIGURE 1. Note the bright specific fluorescence of the colloid material in the follicle at the lower portion of the picture, as well as the specific cytoplasmic fluorescence of the lining cells of the follicle. The nuclei of these cells contrast with the cytoplasm by their lack of fluorescence.

the patients and the controls; the staining was similar to that usually found in experimental and human thyroiditis (FIGURE 2). Immunohistochemical results such as these indicate that different and, perhaps, multiple tissue antibodies may be elicited in patients whose clinical and pathological manifestations are similar and, on the other hand, they indicate that similar tissue-localizing antibodies may be elicited in patients with differing clinicomorphologic manifestations. Despite the presumptive evidence that an autoimmunity mechanism is the basis for the above-described clinical and pathological syndrome in our patients, it is entirely possible that a circulating antibody with faculties of specific tissue localization is not instrumental in the pathogenesis of the disease.

A final comment may be made concerning the study of the hypersensitive state and diseases associated with it. There is little doubt that the last years have witnessed a remarkable progress in the understanding of hypersensitivity and that numerous facts have been uncovered with the help of newly developed techniques. However, it is realized that the use of these techniques also has uncovered many new and still unsolved problems that put us far from any final answers.

References

1. COONS, A. H. & M. H. KAPLAN. 1950. Localization of antigen in tissue cells: improvements in method for detection of antigen by means of fluorescent antibody. J. Exptl. Med. **91:** 1.
2. COONS, A. H., E. H. LEDUC & J. M. CONNOLLY. 1955. Studies on antibody production: I. A method for the histochemical demonstration of specific antibody and its application to a study of the hyperimmune rabbit. J. Exptl. Med. **102:** 49.
3. MELLORS, R. C. 1959. Fluorescent-antibody method. *In* Analytical Cytology, 1. R. C. Mellors, Ed. McGraw-Hill. New York, N. Y.
4. DIXON, F. J., J. J. VAZQUEZ, W. O. WEIGLE & C. G. COCHRANE. 1958. Pathogenesis of serum sickness. A.M.A. Arch. Pathol. **65:** 18.
5. DIXON, F. J., J. J. VAZQUEZ, W. O. WEIGLE & C. G. COCHRANE. 1959. *In* Cellular and Humoral Aspects of the Hypersensitive States. H. S. Lawrence, Ed. Hoeber-Harper. New York, N. Y.
6. MCLEAN, C. R., J. D. FITZGERALD, O. Z. YOUNGHUSBAND, & J. D. HAMILTON. 1951. Diffuse glomerulonephritis induced in rabbits by small intravenous injections of horse serum. A.M.A. Arch. Pathol. **51:** 1.
7. DIXON, F. J., W. O. WEIGLE, J. J. VAZQUEZ, & J. D. FELDMAN. 1960. The immunologic and morphologic consequences of prolonged daily injection of bovine albumin (BSA). Federation Proc. **19:** 205.
8. VAZQUEZ, J. J., J. D. FELDMAN, F. J. DIXON & W. O. WEIGLE. 1960. The morphology and immunology of chronic irreversible glomerulonephritis in rabbits. 57th Ann. Meeting of Am. Assoc. Pathol. and Bacteriol. Proceedings. **:** 16.
9. COCHRANE, C. G. & W. O. WEIGLE. 1958. The cutaneous reaction to soluble antigen-antibody complexes. J. Exptl. Med. **108:** 591.
10. COCHRANE, C. G., W. O. WEIGLE & F. J. DIXON. 1959. The role of polymorphonuclear leukocytes in the initiation and cessation of the Arthus vasculitis. J. Exptl. Med. **110:** 481.
11. DIXON, F. J. 1953. The use of I^{131} in immunologic investigation. J. of Allergy. **24:** 547.
12. MCKINNON, G. E., E. C. ANDREWS, R. H. HEPTINSTALL & F. G. GERMUTH. 1957. An immunohistologic study on the occurrence of intravascular antigen-antibody precipitation and its role in anaphylaxis in the rabbit. Bull. Johns Hopkins Hosp. **101:** 258.
13. VAZQUEZ, J. J. & F. J. DIXON. 1958. Immunohistochemical analysis of lesions associated with "fibrinoid change." A.M.A. Arch. Pathol. **66:** 504.
14. MELLORS, R. C. & L. G. ORTEGA. 1956. Analytical pathology. III. New observations on the pathogenesis of glomerulonephritis, lipoid nephrosis, periarteritis nodosa and secondary amyloidosis in man. Am. J. Pathol. **32:** 455.
15. FENNELL, R. H., J. J. VAZQUEZ & C. R. REDDY. An immunohistochemical study of the renal lesions of progressive systemic sclerosis and malignant hypertension. To be published.

16. Vazquez, J. J. & F. J. Dixon. 1956. Immunohistochemical analysis of amyloid by the fluorescence technique. J. Exptl. Med. **104:** 727.
17. Dixon, F. J. & J. J. Vazquez. 1959. Immunohistochemical analysis of hypersensitivity and related lesions. *In* Mechanisms of Hypersensitivity. : 191. J. H. Shaffer, G. A. LoGrippo and M. W. Chase, Eds. Little, Brown. Boston, Mass.
18. Dixon, F. J. 1958. Allergy and immunology: autoimmunity in disease. Ann. Rev. Med. **9:** 257.

Discussion

Gustave J. Dammin (*Peter Bent Brigham Hospital, Boston, Mass.*): With reference to the illuminating studies of Jacinto J. Vazquez and the intriguing report by Melvin H. Kaplan, may I mention our documentation of the progression of one of the streptococcal-related diseases in the absence of an episode of clinical infection usually considered a part of the development of rheumatic fever and glomerulonephritis. In 1954, Merrill, Murray, and Harrison carried out the first transplantation of a kidney from a well twin to his identical twin brother who had chronic glomerulonephritis.[1] After good function of the transplant was well established, the patient's own kidneys were removed. In the subsequent five years there was no recognizable streptococcal infection but, during the latter part of last year, proteinuria appeared and there was slight nitrogen retention. Last month a biopsy of the transplanted kidney showed the pattern of early membranous glomerulonephritis with lipid deposits in the glomeruli and tubules.

Reference

1. Merrill, J. P., J. E. Murray, J. H. Harrison & W. R. Guild. 1956. Successful homotransplantation of the human kidney between identical twins. J. Am. Med. Assoc. **160:** 277.

Bernard M. Wagner (*University of Washington, Seattle, Wash.*): The localization of proteins in vascular and connective tissue fibrinoid by the immunofluorescent technique has been of value but represents only one type of approach. Careful histochemical studies show that fibrinoid varies in its physicochemical properties in the same disease. A heterogeneous group of fibrinoids exists and their reactivity is dependent on the nature of the disease, rate of formation, and age of the process. The variance in results from various laboratories as regards the localization of gamma globulin and/or fibrin in fibrinoid may be attributed to these factors.

AMYLOIDOSIS: PRELIMINARY CLINICAL, CHEMICAL, AND EXPERIMENTAL OBSERVATIONS*

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Although it has been an object of clinical, pathological and, more recently, chemical studies over the course of the past seventy-five years, amyloidosis remains one of the more intriguing enigmas in medicine. As a subject for clinical investigation, this disease is appealing for several reasons. First, it can be produced experimentally in animals.[1-5] Second, amyloid is accumulated in tissues in sufficient abundance to make its availability for chemical characterization appear a reasonable objective. Third, it is a disease of considerable clinical importance. Thus amyloidosis is a common concomitant of rheumatoid arthritis;[6,7] it is a common cause of death of patients with paraplegia, chiefly because of the severe decubitus ulcers and osteomyelitis that often accompany it;[8] it is the most important single cause of death of patients with leprosy (unpublished observations, in preparation). It often accompanies multiple myeloma and, not infrequently, occurs without known predisposing disease, especially in certain Mediterranean countries where two familial forms are indigenous.[9,10]

Surely a disease with these characteristics should prove an easy one to understand. This has not proved, however, to be the case. The nature of amyloid is still obscure, the reasons for its diverse origins is unknown, the pathogenesis of none of its forms is understood, and means of its prevention and cure are still uncertain.

Approximately six years ago several workers in this laboratory undertook a long-range study of the disease in the hope that what was learned would be helpful in gaining understanding not only of amyloidosis, but also of the even more puzzling diseases of connective tissue that often underlie it. Some of this work has been published, some is in manuscript form, and some is still in progress and permits mention of only the most preliminary conclusions. Nevertheless, as an example of one area in which research in connective tissue is reaching clinical application, a brief and rather general review of the problem may be welcome.

Since our studies have been focused along three lines, clinical, chemical, and experimental, it may be helpful at the outset to emphasize two clinical points.

The first pertains to classification. Amyloidosis is often classified into specific types by rather rigid criteria.[11-13] It is said, for example, that "primary" amyloidosis is characterized by predominant involvement of the mesenchymal tissues (blood vessels, skin, heart, tongue, gastrointestinal tract, and nerves),

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while secondary amyloidosis involves chiefly the spleen, kidneys, liver, and adrenal glands. The primary form is said to exhibit less characteristic staining reactions to crystal violet and Congo red stains. The amyloid accompanying multiple myeloma is said to be similar in distribution and tinctorial characteristics to the "primary" form.

In our opinion these criteria of differentiation of types of amyloidosis present hazards because there are numerous exceptions and overlapping manifestations. For example, patients with amyloidosis without predisposing cause frequently exhibit massive involvement of spleen, liver, and kidneys, and their tissues may stain strongly with crystal violet and Congo red. We believe that the differentiation of primary and secondary amyloidosis should depend largely on the presence or absence of an underlying disease that it is known to predispose to this condition, at least in our present state of knowledge.

The second clinical point pertains to diagnosis. In our hands gingival biopsy has proved to be an extremely helpful aid in confirming the presence of this disease.[14] It gave positive results in 5 of the 6 cases of proven primary amyloidosis that we have studied. It was also positive in approximately 65 per cent of 24 patients with leprosy who were suspected strongly of having this disease (unpublished observations, in preparation). A more detailed review of the diagnosis of amyloidosis has been presented elsewhere.[14a]

Chemical Composition of Amyloid

Initial studies were focused on the isolation and characterization of amyloid. As a beginning, the extraction procedures of Hass and Schultz[15] and Wagner[16] were repeated. It soon became apparent that efforts to extract amyloid were rendered extremely difficult by two factors: first, the marked insolubility of the substance and, second, the lack of an appropriate method of identifying the presence of amyloid in a given extract. Because of the capricious nature of the binding of Congo red to amyloid, it did not seem appropriate to use the presence or absence of this dye as a guide, as Hass had done. Indeed, it was concluded that the only way to demonstrate that a given extract preferentially had removed amyloid would be to demonstrate that a great deal more of an amyloid-laden organ would dissolve in the given solution than would the comparable organ of a normal individual. When the methods of Hass and Wagner were studied from this point of view it was found that as much material, or more, dissolved from the normal organs as from the amyloid-laden tissues.

For this reason, extraction efforts were temporarily suspended, and a method was adopted by which chemical analyses of normal livers and spleens were compared with comparable analyses of livers and spleens so loaded with amyloid that the parenchymal tissue was largely replaced. It was apparent that livers and spleens containing an estimated 50 to 90 per cent amyloid, from patients with both primary and secondary amyloidosis, differed from organs without amyloid in several respects[17] (unpublished observations). There was a higher water content (averaging 80 per cent as opposed to an average normal of 74 per cent), and a higher hexosamine content (averaging 1.0 per cent dry weight as opposed to an average normal of 0.43 per cent dry weight). Paper

chromatographic studies showed that the predominant amino sugar in amyloid-laden tissues was glucosamine, whereas normal livers and spleens contained considerable galactosamine as well. Similarly, in amyloid-laden livers the hexoses consisted of galactose, mannose, and fucose, as well as glucose whereas, in normal livers, sugars other than glucose were present only in trace amounts. There were no differences in the concentrations of nitrogen, uronic acid, hydroxyproline, sulfur, phosphorus, and ash between the normal and the amyloid-containing tissues.

An extract of finely ground amyloid liver was prepared by a method described by Bestetti and associates[17a] involving centrifugation in 2 *M* sucrose at 17,500 rpm (28,000 g) for 10 min. The residue prepared in this manner was metachromatic and stained intensely with Congo red. Analysis of this material indicated that it contained hexosamine in a concentration of 1.64 per cent dry weight, 11.8 per cent nitrogen, 0.32 per cent uronic acid (orcinol method), and 0.73 per cent hydroxyproline. The concentrations of phosphorus and ash were comparable to those of the normal tissues.

These data suggest that amyloid is a hydrophylic substance or substances, predominantly protein (but not collagen), containing glucosamine, galactose, mannose, and fucose. The material does not appear to be an acid mucopolysaccharide; if chondroitin sulfate and heparitin sulfate are present, as suggested by others,[18,19] their concentration must be small indeed.

Casein-Induced Amyloidosis in the Rabbit: General Description and Immunological Studies

Since the chemical studies described above showed little promise of further identifying the nature of amyloid, efforts were next directed to defining an experimental model of this disease that would be suitable for other forms of investigation. Serial injections of 10 per cent sodium caseinate had been shown to be effective in inducing amyloidosis in mice,[2] and it was decided to apply this technique. It was soon found that biweekly injections of 5 cc. of 10 per cent sodium caseinate almost invariably led to the appearance of amyloid in the spleens and kidneys of rabbits.[20,21] Splenic involvement was noted in most of the animals after three months of injections, being rather massive in some. After 4 months nearly all animals exhibited amyloidosis of the spleen. Renal involvement lagged behind splenic involvement by approximately 2 months. Amyloid was noted in the liver in only 20 per cent of the animals, and then only in small amounts. Except for the paucity of amyloid in the liver, the experimental disease was closely analogous to the human disorder. As the disorder developed, the animals lost weight and eventually exhibited nephrosis, with albuminuria, elevated serum cholesterol concentrations, and hypoalbuminemia. Histologically, the experimental tissues appeared identical with those of human patients with this disease.

Blood studies performed on these animals showed that after 2 months of injections there were significant increases in serum concentration of hexosamine and gamma globulin. In 39 rabbits studied, the average normal values were 79 mg./100 ml. and 0.8 gm./100 ml., respectively. After two months of injections the hexosamine and gamma globulin concentrations had risen to 112

mg./100 ml. and 2.0 gm./100 ml., respectively; after four months they were 116 mg./100 ml. and 1.5 gm./100 ml., respectively. Elevations in alpha and beta globulins also were noted, but were less consistent. Serum precipitins to caseinate were demonstrated in most bleedings, but they were in low titer.

Attempts were made to induce amyloidosis in rabbits with much smaller doses of caseinate, comparable to the doses of antigens that are customarily used to induce circulating antibodies. Among these methods were the use of serial intravenous, subcutaneous, and intradermal injections of 0.8 per cent sodium caseinate, and twice-weekly intravenous injections of an alum precipitate of sodium caseinate (0.2 per cent). Although in occasional rabbits minimal amyloidosis developed after 5 to 8 months, an occasional control animal receiving serial injections of isotonic saline likewise did so. None of these experiments yielded results that compared in consistency or degree with those of animals injected with 0.5 cc. of 10 per cent caseinate twice weekly. On the other hand, the subcutaneous administration of 2.5 cc. of 1 per cent caseinate in amphogel every two weeks for from three to seven months led to massive amyloidosis in the three animals studied.

It is of interest that only the methods that ultimately led to a high incidence of amyloidosis were successful in inducing demonstrable circulating precipitating antibodies or elevations in gamma globulin concentrations of the magnitude mentioned above. Transient elevations in serum hexosamine concentration to levels as high as 180 mg./100 ml. were noted after a few of the intravenous injections in animals that did not develop amyloid.

Is Amyloid an Antigen-Antibody Precipitate?

Data of the sort summarized above and other observations reported in the literature[22] suggest that there is a close relationship between immunological mechanisms and the pathogenesis of amyloidosis. It is in many ways attractive to postulate, as others have done,[23] that amyloid represents the *in vivo* accumulation of antigen-antibody precipitate. Efforts have been made in this laboratory to test this hypothesis by experiments of three sorts.

First, the rate of uptake of I^{131}-labeled sodium caseinate into the amyloid-laden spleen was determined following its subcutaneous injection into animals with casein-induced amyloidosis and into normal controls. There was no difference in uptake of I^{131} by the spleen or in the serum between the amyloid and the normal rabbits.

Second, the biological half life of I^{131}-labeled gamma globulin was determined in 3 patients with amyloidosis, as compared with 4 individuals with rheumatoid arthritis and 3 controls (J. Mills, E. Calkins, and A. S. Cohen, unpublished observations). The biological half life was normal (21 days) in 1 patient with primary amyloidosis (without nephrosis), it was normal (14 days) in 1 patient with severe rheumatoid arthritis who subsequently developed amyloidosis over the ensuing 6 months, it was only slightly shortened (11 days) in the latter patient after his amyloidosis (with mild nephrosis) had developed, and it was much shortened (4.5 days) in 1 patient with advanced primary amyloidosis and massive nephrosis. It was apparent that the known propensity of nephrosis per se to be associated with a decreased biological half life of gamma globulin made the effect of amyloidosis per se difficult to assess. One could not

conclude, however, that there was a shortened biological half life in all patients with amyloidosis. It is of interest that 3 of the 4 patients with rheumatoid arthritis exhibited normal curves. The fourth, the only one with large amounts of rheumatoid factor and demonstrable S.21 gamma globulin in the serum, had a definitely shortened biological half life (8.5 days).

The third set of experiments involved the immunochemical quantitation of gamma globulin in the amyloid-laden livers and spleens of human patients, performed in collaboration with David Gitlin.[24] Gitlin had previously demonstrated that when gamma globulin, as antibody, is combined with antigen in a specific precipitate, the gamma globulin also will combine with its own specific antibody, although only to approximately 30 per cent of the efficiency it alone had previously exhibited. It was reasoned that if amyloid were indeed gamma globulin, combined with an unknown antigen as a specific precipitate, the same property should hold true. Finely ground specimens of amyloid-laden human livers and spleens therefore were incubated with rabbit antihuman gamma globulin. The amount of unreacted gamma globulin then was determined by combining it with a known amount of human gamma globulin. By this means it was possible to demonstrate, in unwashed livers and spleens, the amount of gamma globulin that would have been anticipated from the contained serum (for two normal livers, 1.8 and 2.8 mg./gm. wet weight; for two amyloid-laden livers,* 0.25 and 3.7 mg./gm. wet weight; for two normal spleens, 2.1 and 8.3 mg./gm. wet weight; for two amyloid-laden spleens, 4.5 and 5.6 mg./gm. wet weight). The gamma globulin present in blood and extracellular fluid was then removed by extensive washing with saline. Following washing, the residual material of the amyloid tissues (60 per cent of the original weight) stained intensely with crystal violet and Congo red. None of the 9 normal residues contained demonstrable gamma globulins, nor did the residues from the two amyloid-laden livers. The residues from the amyloid-laden spleens contained 0.03 and 0.1 mg./gm. wet weight (original). Neither of these amounts appeared sufficient to justify the conclusion that gamma globulin comprised a major component of the amyloid.

None of these three experiments, therefore, provided support for the suggestion that amyloid is an antigen-antibody precipitate. On the other hand, the results of the Coons fluorescent staining technique in the hands of Mellors, and Ortega[25] and Vazquez and Dixon[23] have shown that gamma globulin is preferentially bound in the sites of amyloid accumulation in both humans and rabbits with this disorder. Obviously, three negative experiments do not negate the results of one positive experiment. The authors suggest, however, that it is entirely possible that trace amounts of gamma globulin and other substances may be bound to amyloid, as is Congo red, for instance. The question whether amyloid is comprised of gamma globulin remains open at this date.

Anatomic and Structural Studies of Amyloid

In the course of the detailed histological studies of experimental amyloidosis in the rabbit[21] it was noted that amyloid was birefringent with polarized light. This observation, which also had been made by Missmahl and Hartwig,[26] sug-

* One patient was nephrotic and had decreased serum gamma globulin concentration.

gested that the substance had a greater structural organization than previously had been believed. In order to explore this possibility and to determine more precisely the earliest site of amyloid accumulation, electron microscope studies were undertaken.[33] Tissues were fixed in Dalton's solution and buffered osmium tetroxide in sucrose. The results showed that, while amyloid appeared in close proximity to the basement membrane of the spleen and kidney[27] of the rabbits, it usually could be differentiated from this membrane and appeared separate from it. The amyloid was always separated from the vascular bed by an endothelial lining. The amyloid exhibited a finely filamentous appearance, the filaments being much thinner than collagen and without the characteristic 640 Å periodicity of collagen. This filamentous appearance was characteristic of amyloid in both the spleen and kidney of the rabbits and in the kidney, liver, trachea, and skin of human patients with both primary and secondary amyloidosis.[28] The nature of these fibrils and their possible relationship to the pathogenesis of amyloidosis are not understood, and are currently under investigation.

The Effect of Variables on the Course of Experimental Amyloidosis

The availability of a well-defined experimental model of amyloidosis provides an excellent opportunity for the assessment of possible therapeutic measures as well as of variables of theoretical interest. To date the effect of 3 variables has been studied, thus far only in a preliminary fashion.

The first is the effect of cortisone. Teilum[29] noted that in his mice rendered "preamyloidotic" by previously administered casein the administration of cortisone was followed by a marked acceleration in the rate of development of amyloidosis. This observation is of importance to clinicians because of the possible implications of administering corticoids to patients with rheumatoid arthritis, which already is known to predispose to this disease. In a preliminary study in this laboratory, however, 18 rabbits were given caseinate (0.5 gm. subcutaneously twice weekly) for 5 months. Eleven of these animals were given cortisone (5 mg./kg. 6 days per week) during the last 3 months of the injections. A comparison of the 11 cortisone-treated rabbits with the 7 casein-injected controls revealed that the incidence of amyloidosis appeared to decrease slightly as a result of cortisone therapy. It could certainly be stated that the cortisone did not accentuate the disease.

The next variable studied was splenectomy. This study was undertaken because Pirani *et al.*[30] had noted in their casein-injected mice that the incidence and extent of amyloidosis in the kidneys of splenectomized mice was considerably less than that of nonsplenectomized animals. This observation suggested the possibility that amyloid is synthesized in the spleen and subsequently deposited in the kidneys. The incidence of amyloidosis of the kidney and liver in 6 splenectomized casein-injected rabbits therefore was compared with that in 12 casein-injected controls. Contrary to expectations, the incidence and extent of renal amyloidosis appeared slightly increased in the splenectomized animals. Amyloidosis of the liver, noted in only 1 of the 12 control animals, was seen in 3 of the 6 splenectomized animals. Furthermore, splenectomy appeared to accelerate the hexosamine elevation following the onset of

the caseinate injections. Thus in the control animals the average hexosamine concentration after 3 weeks was 98 mg./100 ml. (S.D., 10.2; average initial value 80, S.D. 9.0). In the splenectomized animals the average hexosamine concentration after 3 weeks was 119 mg./100 ml. (S.D., 12.6; average initial value, 77, S.D. 12.8). After 3 months the average hexosamine concentrations in the two groups were 111 and 120 mg./100 ml., respectively. The reason for the apparent alteration in hexosamine response after splenectomy is not clear. While it is true that different species were used, other factors may also have contributed.

The third variable studied was the effect of β-aminoethylisothiouronium bromide (AET). The use of this agent, a powerful sulfhydryl donor, was suggested by the observation that during the course of caseinate injections in the rabbits there is a fall in serum-free sulfhydryl concentration, from initial average values of 38 μmole/100 ml. (S.D., 6.7) to 23 μmole/100 ml., (S.D., 1.7) after 8 to 9 weeks of injections. This fall in serum-free SH concentration could be prevented by the oral administration of AET (100 mg. per rabbit per day). In initial experiments, however, it was not possible to prevent the development of amyloidosis by this means.

Clinical Studies

The ultimate objective of any studies of amyloidosis is to gain more understanding of the disease in humans and, from this, to devise methods of prevention and cure. Throughout the conduct of the project we have regarded results in rabbits as exploratory only, until they can be demonstrated in human beings. The chemical studies reported above were done primarily on clinical material. The histological and electron microscopy observations have been made on material from patients as well as from rabbits. The observations of the relationship of prolonged hyperglobulinemia and hyperhexosaminemia to the induction of amyloidosis in rabbits are difficult to correlate with clinical material because of the long follow-up that would be required. Observations on the sera of 8 patients with proved amyloidosis secondary to various inflammatory diseases, as opposed to those on 9 individuals with so-called primary amyloidosis have shown several points of interest.[31] All patients in both groups, with 1 exception, exhibited increased serum hexosamine concentrations, with average values of 151 and 157 mg./100 ml. respectively (normal, 75 mg./100 ml.; with S.D. 11.9). This was true both of the patients with nephrosis (in whom it would be expected) and of those without nephrosis. Four of the patients with primary amyloidosis exhibited C-reactive protein reactions of 4+, as did 1 of the 4 patients with secondary amyloidosis on which this test was done. Serum electrophoretic patterns showed elevations in alpha and beta globulins in many instances. Few of the patients had hypergammaglobulinemia, although it must be borne in mind that by the time the diagnosis had been made most of the patients had far-advanced disease.

The patient with primary familial amyloidosis of the sort described by Andrade[9] and Rukavina *et al.*,[32] had a normal hexosamine concentration and negative C-reactive protein reaction.

These observations suggest either that amyloid per se may act as an inflam-

matory stimulus—which appears unlikely in view of the lack of cellular infiltration around accumulations of this substance—or that primary amyloidosis is associated with a metabolic abnormality that mimics the changes accompanying chronic inflammation. Another possible explanation is that the patients assumed to have primary amyloidosis had, in fact, undiagnosed inflammatory disease. Thorough clinical study of each "primary" case, however, failed to provide evidence of such a disease.

Another area where clinical investigation has revealed results of some interest pertains to the relationship between amyloidosis and the latex fixation test. Since both are presumably associated with protein abnormalities, one reasonably might anticipate an association between the two. The latex fixation test results proved to be negative in all of the 17 patients with primary or secondary amyloidosis in our clinic. On the other hand, preliminary studies of sera from 100 patients with leprosy, conducted in collaboration with members of the United States Public Health Service Hospital at Carville, La., indicated, first, that there is a high incidence of positive latex reactions in this disease and, second, that there is a higher incidence of amyloidosis in patients showing positive test results than in those showing negative ones (E. S. Cathcart, R. Williams, Jr., E. Calkins, and A. S. Cohen, in preparation). Only a few of the leprosy patients whose sera reacted in the latex test had rheumatoid arthritis. Further implications of this observation are currently under study.

Conclusions

Amyloid is a hydrophylic protein or proteins containing a significant moiety of carbohydrate, chiefly glucosamine, galactose, mannose, and fucose. It may contain some lipid. It is not collagen.

Amyloidosis can be induced in rabbits by the subcutaneous administration of 5 cc. of 10 per cent sodium caseinate twice weekly. The experimental disease is closely analogous to the human disorder.

Amyloidosis in rabbits is preceded by a period of increased serum concentrations of hexosamine and gamma globulin and by a decrease in serum-free sulfhydryl groups.

Although casein appears to induce amyloidosis by virtue of an immunological process, thus far evidence from this laboratory is opposed to the conclusion that amyloid is an antigen-antibody precipitate.

Amyloid, when fixed with osmic acid, has a characteristic fibrillar appearance. This appearance has been noted in all specimens of amyloid thus far studied, both in rabbits and in humans with primary and secondary disease.

Amyloidosis in rabbits, induced by casein, is not accentuated by cortisone, nor is it inhibited by prior splenectomy.

Amyloidosis in humans, both secondary and primary (primary familial amyloidosis excepted), is accompanied by an increased serum hexosamine concentration.

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References

1. Bailey, C. H. 1916. The production of amyloid disease and chronic nephritis in rabbits by repeated intravenous injections of living colon bacilli. J. Exptl. Med. **23:** 773.
2. Eklund, C. M. & H. A. Reimann. 1936. The etiology of amyloid disease; with a note on experimental renal amyloidosis. A.M.A. Arch. Pathol. **21:** 1.
3. Pirani, C. L., G. B. Bly, K. Sutherland & F. Chereso. 1949. Experimental amyloidosis in the guinea pig. Science. **110:** 145.
4. Rothbard, S. & R. F. Watson. 1954. Amyloidosis and renal lesion induced in mice by injection with Freund-type adjuvant. Proc. Soc. Exptl. Biol. Med. **85:** 133.
5. Richter, G. W. 1956. Alterations in serum globulins during the formation and resorbtion of amyloid in rabbits. J. Exptl. Med. **104:** 847.
6. Teilum, G. & A. Lindahl. 1954. Frequency and significance of amyloid changes in rheumatoid arthritis. Acta. Med. Scand. **149:** 449.
7. Missen, G. A. K. & J. D. Taylor. 1956. Amyloidosis in rheumatoid arthritis. J. Pathol. and Bacteriol. **71:** 179.
8. Dietrick, R. B. & S. Russi. 1958. Tabulation and review of autopsy findings in 55 paraplegics. J. Am. Med. Assoc. **166:** 41.
9. Andrade, C. 1952. A peculiar form of peripheral neuropathy; familial atypical generalized amyloidosis with special involvement of peripheral nerves. Brain. **75:** 408.
10. Heller, H., E. Sohar & L. Sherf. 1958. Familial Mediterranean fever. Arch. Intern. Med. **102:** 50–71.
11. Dahlin, D. C. 1949. Primary amyloidosis, with report of 6 cases. Am. J. Pathol. **25:** 105
12. Dahlin, D. C. 1949. Secondary amyloidosis. Ann. Intern. Med. **31:** 105.
13. Reiman, H. A. & C. M. Eklund. 1935. Long continued vaccine therapy as a cause of amyloidosis. Am. J. Med. Sci. **190:** 88.
14. Trieger, N., A. S. Cohen & E. Calkins. 1959. Gingival biopsy as a diagnostic aid in amyloid disease. Arch. Oral. Biol. **1:** 187.

14a. Calkins, E. & A. S. Cohen. 1960. Diagnosis of amyloidosis. Bull. Rheumat. Dis. (April).

15. Hass, G. & R. Z. Schultz. 1940. Amyloid I. Methods of isolating amyloid from other tissue elements. A.M.A. Arch. Pathol. **30:** 240.
16. Wagner, B. 1955. Histochemical studies of fibrinoid substances and other adrenal tissue proteins IV. Protein character of amyloid. A.M.A. Arch. Pathol. **60:** 221.
17. Giles, R. B., Jr. & E. Calkins. 1955. Study of the composition of secondary amyloidosis. J. Clin. Invest. **34:** 1476.

17a. Bestetti, A., C. L. Pirani & H. R. Catchpole. Thesis of the University of Illinois.

18. Hass, G. 1942. Studies of amyloid II. The isolation of a polysaccharide from amyloid-bearing tissues. A.M.A. Arch. Pathol. **34:** 92.
19. Meyer, K. 1958. Heparitin sulfate. Federation Proc. **17:** 264.
20. Giles, R. B., Jr. & E. Calkins. 1958. The relationship of serum hexosamine, globulins, and antibodies to experimental amyloidosis. J. Clin. Invest. **37:** 846.
21. Cohen, A. S., E. Calkins & C. I. Levene. 1959. Studies on experimental amyloidosis. I. Analysis of histology and staining reactions of casein-induced amyloidosis in the rabbit. Am. J. Pathol. **35:** 971.
22. Letterer, E. 1949. Some new aspects of experimental amyloidosis. J. Pathol. Bacteriol. **61:** 496.
23. Vazquez, J. J. & F. J. Dixon. 1956. Immunohistochemical analysis of amyloid by the fluorescence technic. J. Exptl. Med. **194:** 727.
24. Calkins, E., A. S. Cohen & D. Gitlin. 1958. Immunochemical determinations of gamma globulin content of amyloid. Federation Proc. **17:** 431.
25. Mellors, R. C. & L. G. Ortega. 1956. Analytical pathology III. New observations on the pathogenesis of glomerulonephritis lipid nephrosis, periarteritis nodosa, and secondary amyloidosis in man. Am. J. Pathol. **32:** 455.
26. Missmahl, H. P. & M. Hartwig. 1953. Polarisationsoptische Untersuchungen an der Amyloidsubstanz. Arch. pathol. Anat. u. Pathol. Virchow's. **324:** 489.
27. Cohen, A. S. & E. Calkins. 1959. A light and electron microscopy study of human and experimental amyloid disease of the kidney. Arthritis and Rheumatism. **2:** 70.
28. Cohen, A. S. & E. Calkins. 1959. Electron microscopic observations on a fibrous component in amyloid of diverse origins. Nature. **183:** 1202.
29. Teilum, G. 1952. Cortisone-ascorbic acid interaction and the pathogenesis of amyloidosis. Mechanism of action of cortisone on mesenchymal tissues. Ann. Rheumatic Diseases. **2:** 119.
30. Pirani, C. L., H. R. Catchpole & O. Moore. 1959. Prevention of casein induced amyloidosis by splenectomy. Federation Proc. **18:** 500.
31. Calkins, E. & A. S. Cohen. 1959. Similarity of serum protein changes in primary and secondary amyloidosis. J. Clin. Invest. **38:** 993.

32. Rukavina, J. G., W. D. Block, C. E. Jackson, H. F. Falls, J. H. Carvey & A. C. Curtis. 1956. Primary systemic amyloidosis. Medicine. **35:** 239.
33. Cohen, A. S., L. Weiss & E. Calkins. Observations on the fine structure of the spleen in experimental amyloidosis in rabbits. Am. J. Path. In press.

Discussion

Gustave J. Dammin (*Peter Bent Brigham Hospital, Boston, Mass.*): This comprehensive report answers many questions about amyloidosis. I am curious to know about the experimental animals and the cellular pattern in the marrow, spleen, and lymph nodes during the development of amyloidosis. In the spleen which you discussed, the lymphoid follicle was presumably atrophic. If amyloid is a cell product, one would expect plasmacytosis, perhaps in the marrow.

Calkins: Our histological studies are not yet complete enough to answer that question.

GLOMERULAR EXTRINSIC MEMBRANOUS DEPOSIT WITH THE NEPHROTIC SYNDROME*

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The increasing use of serial renal biopsies in clinical medicine presents an unusual opportunity to study the ground substance of the glomerulus and to correlate morphologic and clinical changes. The presence of proteinuria and, frequently, of the nephrotic syndrome provides a means of selecting patients with potential lesions of the ground substance.

The most striking lesion of the ground substance seen in this study of the glomerulus is the extrinsic membranous deposit (EMD). This lesion consists of basement membrane changes with diffuse accumulations of protein material that distort the epithelial cell. A membranous lesion of this type described by Jones,[1,2] who used light microscopy, was classified as a form of chronic glomerulonephritis. Churg and Grishman[3] interpreted the lesion as the result of the proteinuria and not specifically pathognomonic of a disease entity.[7] Farquhar[4] and Movat and McGregor[5] added electron-microscopy findings, but classified their cases as an adult form of lipoid nephrosis. In our series of 187 biopsies with successful electron microscopy in 74 per cent, 6 cases of EMD were diagnosed. We believe that the EMD and its associated clinical findings are a special subgroup of the nephrotic syndrome and that it is probably closely related to, or a variant of, systemic lupus.

Methods

Percutaneous renal biopsies were performed, using the technique previously described.[6] Several 1-mm. cubes of renal cortex were immersed quickly in ice-cold 1 per cent osmic acid solution buffered at *p*H 7.35 with Veronal buffer.[7] Fixation was for a 1-hour interval and was followed by dehydration through alcohols. The tissue was embedded in a mixture of *n*-butyl and methyl methacrylate polymerized by ultraviolet light. The glomeruli were identified in thick sections stained with Azure blue, and the glomeruli alone were sectioned at about 100 Å on a Servall microtome. These were mounted on 100-mesh copper grids covered with Formvar film and examined with an RCA EMU-3D. Material for light microscopy was fixed in Helly's fluid, paraffin-embedded, and serial-sectioned, and alternate sections were stained with hematoxylin and eosin, paS Alcian blue, and by the Mallory-Heidenhain method.

A number of standard procedures for the clinical investigation of the cases with EMD were carried out as described in the clinical histories. In addition, repeated searches were made for lupus erythematosus (L.E.) cells in preparations of the peripheral blood cells.[8] Antistreptolysin O (ASO) titers were determined on occasion. At every clinic visit a complete urinalysis was carried out, with occasional electrophoresis of the urinary proteins. Blood pro-

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teins were measured by the standard Howe precipitation techniques and by paper electrophoresis with Veronal buffer at *p*H 8.6, using the Spinco Analytrol. Special tests made on these patients will be the subject of a forthcoming report.

Observations

Although 4 of the patients showed no sign of systemic disease, the other 2 had systemic manifestations resembling the systemic lupus syndrome (cases 1 and 2).

Case 1

This white woman, born in Italy in 1922, appeared in good health until December 1951 when, after an attack of tonsillitis, she was given penicillin. Three or 4 days later the joints in her hands, wrists, and ankles became painful and swollen. This joint pain lasted for 3 or 4 days.

In March 1952 she developed a sensation of heaviness in the chest and a fever. She was hospitalized for six weeks with a diagnosis of rheumatic fever and was kept on complete bed rest for the following seven months.

Serial electrocardiograms during this period were said to show nonspecific myocardial changes, and the chest X rays were said to show a 100 to 150 per cent enlargement of the heart. Fluid was aspirated from the thorax. She was found to have a generalized lymphadenopathy, although there was no swelling or pain of the joints.

Eight months prior to her first visit to our clinic in 1955 she had had a recurrence of hot red swollen metacarpal phalangeal joints and, to some extent, of the distal interphalangeal joints. Treatment with cortisone relieved these symptoms, which returned, however, when cortisone was discontinued. In June 1955, after sunbathing, she developed a rash on her upper chest and face. This rash led to the diagnosis of systemic lupus.

Physical Examination

The patient was a well-nourished and well-developed woman in no distress from the confluent erythematous rash described. There was moderate nontender lymphadenopathy. The liver was palpable four fingerbreadths below the right costal margin, but the spleen was not palpated. There was a puffy swelling of both hands, including the fingers and wrists, which the patient thought had been a lifetime abnormality. There was no tenderness of the joints of the hand. There was a 2+ pitting ankle edema.

Laboratory Data

The white blood count was 5250; the hemoglobin was 10.8 gm./100 ml.; the sedimentation rate by the Wintrobe method was 61 mm./hour, corrected to 34; the urine showed 1 to 2 red blood cells per high-power field and no protein; plasma albumin was 3.7 gm./100 ml.; globulin was 4.1 gm./100 ml.; urea clearance was 41 cc./min. by the maximum flow formula; ASO titer was negative; C-reactive protein was 1+; LE cells were seen in preparations of the peripheral blood. The heart was 22 per cent oversized (shown by X ray). During her first hospitalization she had a small temperature rise, and the skin rash gradu-

ally faded. She was given 10 mg. prednisone 3 times a day; this provided great relief of her joint pains and fever. She remained well for the subsequent 4 years on small doses of Meticorten and salicylates.

A second hospitalization was occasioned in 1959, after the patient had become more active. She had noted increased exertional dyspnea, a persistent ankle edema, and a steady gain in weight that did not disappear with the administration of chlorothiazide. On admission, her blood pressure was 113/60 mm. Hg; the pulse was regular at 84/min.; her temperature was normal. There was a noticeable butterfly rash over the face. A soft systolic murmur was heard over most of the precordium, being loudest over the base of the heart, and radiating to the right side of the neck. Multiple small lymph nodes were palpated in the cervical area, but these were not tender. There was a slight enlargement of the heart. Both legs had 2+ pitting edema extending to the thighs, and there was a slight sacral edema.

Laboratory Data

The white blood count was 5000; the hemoglobin was 10.9 gm./100 ml.; the sedimentation rate was 62 mm./hour; the platelet count was 145,000; there were 1.2 per cent reticulocytes; the bleeding and clotting times were normal; the urine contained protein, occasionally hyaline, cellular, and granular casts, numerous white cells, and doubly refractile fat bodies. The serum albumin was 1.5 gm./100 ml.; the globulin was 3.6 gm./100 ml.; the urea clearance was 17 cc./min. (square root formula); the total cholesterol was 360 mg./100 ml. The patient had consistently positive L.E. preparations of the peripheral blood.

The electrocardiogram was normal at this time, but the chest X ray showed a cardiomegaly of 20 per cent. Serial urine protein determinations demonstrated a 24-hour output ranging from 7 to 18 gm. On bed rest, the patient lost 7 kg. of body weight. A percutaneous renal biopsy was carried out without incident, and she was discharged on hospital day 19, improved.

Renal Biopsy. The glomeruli are uniformly involved. A mild glomerular hypercellularity is due to endothelial cell proliferation. The capillary walls are thickened and show a "wire-loop" appearance by light microscopy. Electron photomicrographs of the glomeruli localize the change responsible for the capillary thickening at the area between the basement membrane and the epithelial cell. The basement membrane in the areas of thickening is less dense and has poorly defined margins and, occasionally, droplets and vacuoles. The outer surface of the basement membrane is covered in these areas by an osmophilic material that separates the foot processes of the epithelial cells and frequently separates the epithelial cell and the basement membrane.

The glomeruli contain hyaline thrombi, focal necrosis, and hematoxylin-stained bodies. There are synechiae over the most severely damaged lobules. The cortical tubular pattern is slightly distorted; tubular cells are enlarged and contain hyaline and fatty droplets. There are a few granular and hyaline casts. Small focal interstitial infiltrations consist of lymphocytes. The arterioles and small arteries appear normal.

The electrophoretic pattern of the serum is shown in FIGURE 1. At the time of this report the albumin has diminished to 4.3 gm./24 hours, and the serum electrophoretic pattern has returned to normal.

Comment

As adjudged by the usual criteria, this case belongs to the disease spectrum called systemic lupus erythematosus. If, however, this patient never had had a severe exposure to sunlight and thereby the cutaneous rash, then the only direct evidence of the presence of systemic lupus would have been the L.E. cells in the peripheral blood. At one time she had been examined and treated for 7 months for acute rheumatic fever. Another point of interest is that this patient, despite a rather long illness, has had a remarkably benign course.

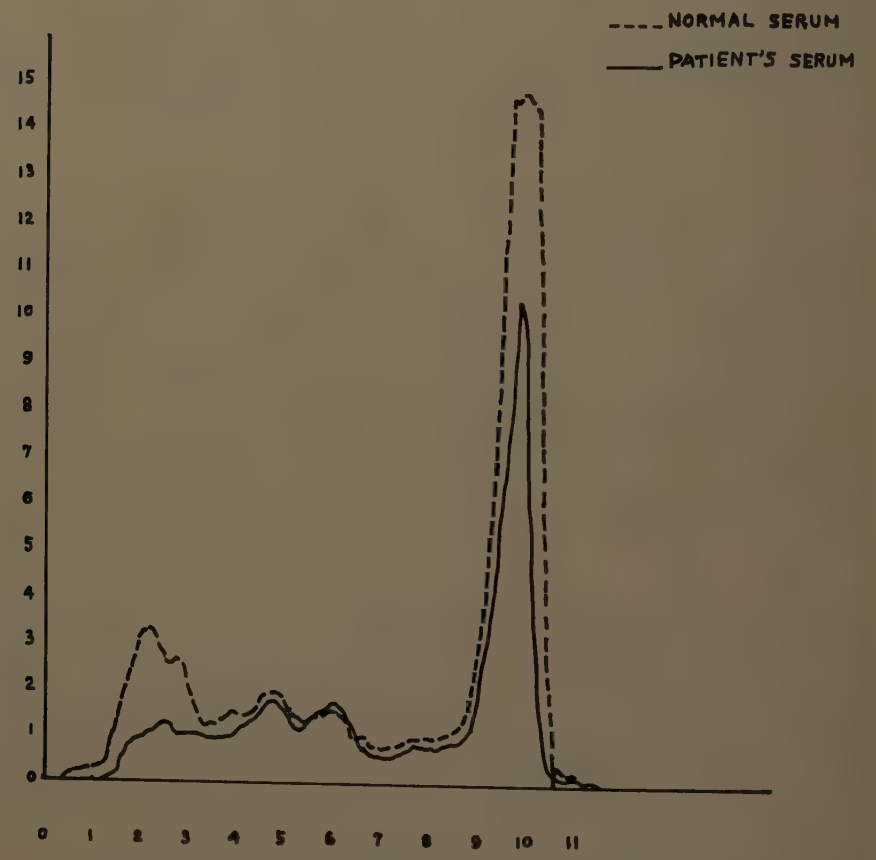

FIGURE 1. Electrophoresis of serum from patient 1.

CASE 2

This white married woman was born in 1908. For more than 15 years prior to her hospital admission in 1959 she had been troubled with persistent arthritis, varying from a mild arthralgia to acute, swollen, tender, hot joints; the symptoms lasted for 3 or 4 days. She had swelling of the joints and 2½ years ago she took cortisone, but stopped it because of nausea.

Shortly after this she had a marked diminution of vision in her right eye, and now is found to have optic atrophy. Some 2 months prior to admission in 1959 she developed anasarca and gained 12 lb. in several days. She had an albuminuria and did not respond to diuretics. A few weeks prior to admission she was found to have right flank pain that spread to the anterior chest. She gave a history of pleurisy 2 years prior to this admission. There was no history of fever or sun sensitivity, but she has an allergy to penicillin and sulfonamides.

Physical Examination

Right optic atrophy was noted. There was some fusiform swelling of the fingers, impaired motion of the left wrist, and swelling of the right knee. There was also a trace of pitting edema of the extremities below the knee.

Laboratory Data

The urine showed albumin and occasional granular casts, a few broad casts, a few red cells, and 25 to 30 white blood cells per high-power field, with doubly refractile fat in the cells; the sedimentation rate, Wintrobe-corrected, was 44 mm./hour; the hemoglobin was 12.7 gm./100 ml.; the red blood cell count was 3.7 million; the white cell count was 8100; the platelet count was 198,000; the bleeding time, clotting time, prothrombin time, and clot retraction time all were normal. A chest X ray revealed marked pleural effusion on the right. No LE cells were demonstrable in several examinations of the peripheral blood. A right renal biopsy was performed. The urea clearance was 45 cc./min. by the standard formula; blood urea nitrogen content was normal. Plasma albumin at this time was 1.6 gm./100 ml. and globulin was 3.7 gm./100 ml. Total cholesterol was 390 mg./100 ml., with esters of 275 mg./100 ml. She lost weight on a low-salt diet.

Renal biopsy. Twenty intact glomeruli are present. These are normally cellular and are not enlarged. The most characteristic change is a uniform thickening of the glomerular capillaries that does not result in narrowing or occlusion of the lumen. Electron microscopy of glomerular thickening suggests that the change is primarily in the epithelial cell with accumulation of material obliterating the foot processes at the junction with the basement membrane (FIGURES 2 and 3). The distortion of the epithelial cells is pronounced and, in some situations, cytoplasmic inclusions in these cells extend to the area of the nucleus. The other cytoplasmic particulates of the glomerular epithelial cells are not remarkable. The basement membrane is thickened only slightly. The endothelial cytoplasm in these areas is intact and has normal numbers and sizes of pores. In the area of the lobular stalk, however, the basement membrane is folded and occasionally thickened in at least 50 per cent of the glomeruli. This change results in the distortion of endothelial nuclei, but is accompanied by no proliferative change. The glomerular space contains no debris. There are no fibroepithelial crescents and no periglomerular fibrosis. The tubular pattern is normal except for mild separation not accompanied by deposition of collagen material. Focal accumulations of mononuclear cells are present in 2 or 3 places. These are not associated with focal tubular damage

FIGURE 2. This electron micrograph of a glomerular capillary from patient 2 shows the characteristic appearance of the extrinsic membranous deposit. The basement membrane, while clearly outlined at the upper left, becomes lighter and has vague borders in areas of more severe involvement (BM). There are irregular spikelike extensions into the deposits (INC) that separate the epithelial cytoplasm (EPITH CYT) from the basement membrane. Bowman's space (BOW SP) is not distorted by proliferative changes. The endothelial cytoplasm (END CYT) along the involved basement membrane appears normal. CAP L, capillary lumen. Osmium fixation. ×4000.

FIGURE 3. The less advanced lesion of EMD, also from patient 2, shows the vacuolated widened basement membrane (V), which at one point contains osmophilic droplets (D). The foot processes (FP) of the epithelial cells (EP) are intact in areas where the basement membrane (BM) is less involved. The foot processes are separated by osmophilic material (EMD) and they are fused on the right where the basement membrane change is the most severe. ×10,000.

or occlusion. The tubular lumen contains small amounts of granular material and, occasionally, partially sloughed cells are present. Many of the collecting tubule cells have accumulations of hemosiderin pigment along the luminal borders. Small arteries and arterioles are not remarkable, although a medium-sized artery included in the biopsy at the corticomedullary junction has a marked focal fibrous thickening of the intima.

Comment

This case, while not demonstrating the full gamut or any of the pathognomonic features of systemic lupus, comes very close to falling within the spectrum of systemic lupus erythematosus and is not dissimilar in this respect from the preceding case. The patient has not been seen for follow-up examination.

Case 3

This married white woman was born in 1903. In 1947 she began having edema of the lower extremities, which has become worse since 1953. In 1949 she began having periorbital and malar edema that was worst in the morning. Hematuria had been observed on at least 1 occasion during this time.

Physical Examination

When she was admitted in 1958, her blood pressure was 170/70 mm. Hg with regular pulse of about 82/min. There was 3+ pitting edema of the feet extending to the knees.

Laboratory Data

The urinalysis revealed 10 to 15 red cells per high-power field, 1 to 3 white cells per high-power field, and doubly refractile fat bodies; there was 2+ to 4+ albumin in the urine. Plasma albumin was 2 gm./100 ml.; the globulin was 3.1 gm./100 ml. The plasma albumin during the period of our observations in 1958 reached a low of 1.3 gm./100 ml. and globulin, a high of 4.4 gm./100 ml. Total cholesterol reached a level of 350 mg./100 ml.

Renal biopsy. Two renal biopsies were carried out, one on November 13, 1958, and one on December 19, 1958. The first renal biopsy shows normal-sized glomeruli with regularly thickened capillary loops. Electron photomicrographs confirmed that this thickening was due to an extrinsic deposit of osmophilic material between a poorly limited extrinsic margin of the basement membrane and the badly distorted epithelial cell. Irregular spikes of basement membranelike material extend into the extrinsic membranous deposit. No proliferative changes are seen in the endothelial or epithelial cells. Bowman's space contains granular material. There is focal tubular atrophy, but casts are not conspicuous. There is no evidence of venous occlusion. A repeat biopsy made one month later shows little progression of the glomerular change.

Clinical Course

Following the sudden appearance of neurological symptoms, she was found to have an intracerebral neoplasm. Surgery for this was not successful and it caused her eventual death.

Comment

This case is difficult to analyze because the patient's death from metastatic disease destroyed any possibility of follow-up on, or appraisal of, the life history of the extrinsic membranous lesion of the kidney, or a more detailed examination of the patient's plasma proteins. It is of interest, however, that the patient showed none of the forthright symptoms of systemic lupus.

Case 4

This married white woman was born in 1886. She entered the hospital December 17, 1958, because of a swelling of the ankles for the preceding 6 months. Her past history had been largely uneventful, except for a photophobia, ready bruising, and joint pains. The character of the joint pains was nonspecific. She was given 20 mg. of prednisone daily for 10 days. At the end of that time her urine was free of albumin, but her edema had increased.

Laboratory Data

On her admission, plasma albumin was 1.8 and 1.6 gm./100 ml. on December 17 and December 26, respectively. Serum cholesterol was 660 mg./100 ml. both times. On December 24 her 24-hour protein output was 3.5 gm.

Renal biopsy. Biopsy performed on December 26 shows thickened glomerular capillary loops, with obliteration of most of the foot processes of the epithelial cells. The deposit outside the basement membrane fuses with a poorly defined outer border. The endothelial cells appear normal, and there is no narrowing of the capillary lumina. The interstitial space is conspicuous, with a diffuse atrophy of many tubules. There is no cellular exudate.

Two peripheral blood preparations were negative for lupus. The patient was discharged from the hospital, and there has been no follow-up.

Comment

It is impossible in this case to eliminate the possibility of systemic lupus or its variants. The history of mild joint pains and photophobia is suggestive, but of no real help in the diagnosis. Moreover, the lack of follow-up makes a resolution of these problems impossible. It is of interest, in speculating on the pathogenesis of the EMD, that at the time of the renal biopsy the patient was losing a moderate amount of protein, 3.9 gm./24 hours.

Case 5

A single white male, born August 25, 1930, was seen in December 1956. He complained of fatigue of 6 months' duration and swelling of the lower extremities of 16 months' duration.

The patient was apparently well prior to September 1955, at which time he first noticed the onset of a gradually increasing ankle edema. Treatment with mercurial diuretics gave only temporary relief, and he was hospitalized in June 1956 because of anasarca. Treatment with adrenocorticotropic hormone and cortisone gave relief of this edema. The patient has continued to suffer fatigue. There is no history of rheumatic fever or scarlet fever in his past.

Laboratory Data

Repeated tests for L.E. cells gave negative results. The serologic test result was negative; the sedimentation rate was 13 mm./hour (Wintrobe method); the hemoglobin was 16 gm./100 ml.; the white cell count was 7800; the urine showed 2+ protein, rare hyaline and cellular casts, 3 to 12 red cells per high-

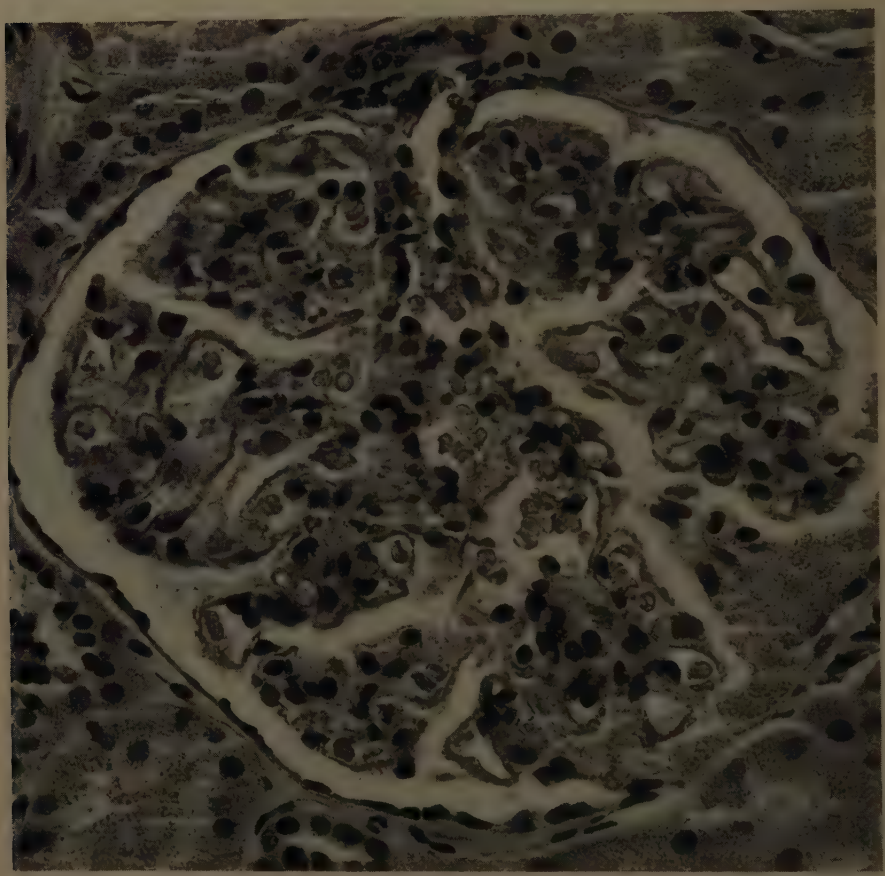

FIGURE 4. Light microscopy of a glomerulus from patient 5 shows the diffuse thickening of the capillaries. Hematoxylin and eosin. ×430.

power field, 0 to 3 white cells per high-power field, with an occasional cast loaded with doubly refractile fat; the urea clearance was 111 cc./min. by the maximum flow formula; ASO titer was 1:100; the serum cholesterol was 350 mg./100 ml.

Renal biopsy. All glomeruli are normal in size and have a uniform appearance. There is a mild endothelial cell proliferation, but the major change is a thickening of the glomerular capillaries (FIGURE 4). The basement membrane is normal in thickness. Separating the basement membrane and the epithelial

cells is a regular thick band of material that occasionally includes portions of the epithelial cells, but occasionally has normally formed foot processes on the outer margin. There are many hyaline droplets in the cytoplasm of the epithelial cells, and an occasional glomerulus has a fibroepithelial crescent. Bowman's capsule and many tubules contain granular debris.

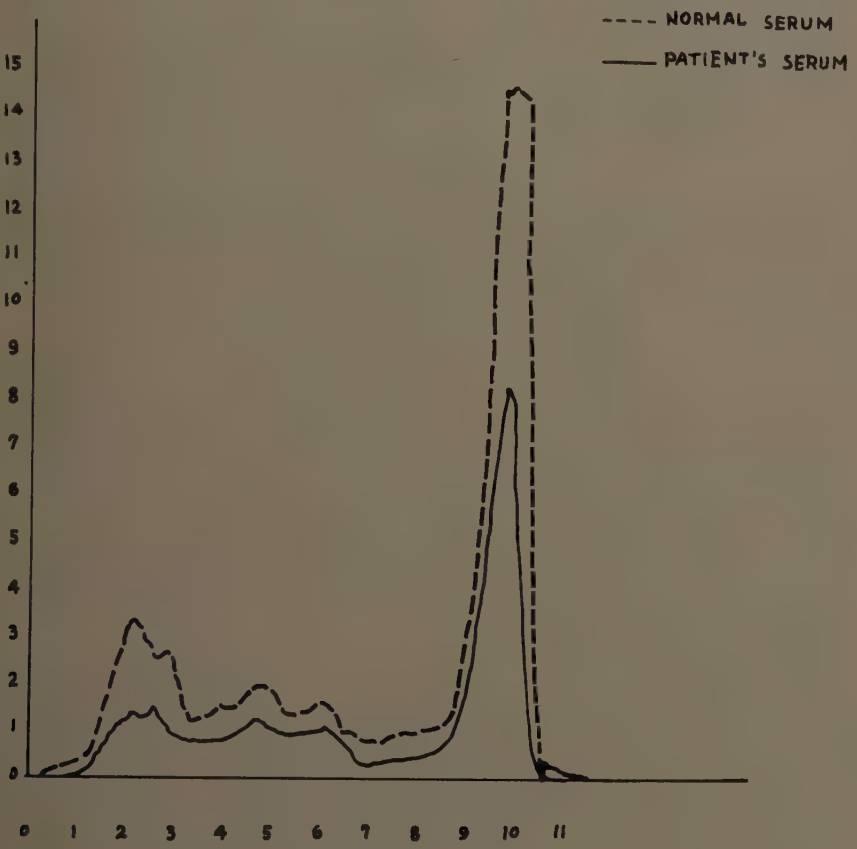

FIGURE 5. Electrophoresis of serum from patient 5.

From the time of his first visit in 1956 until 1960, the patient's plasma albumin ranged from 2.2 to 4 gm./100 ml. The average urine loss was 10 gm./day. In May 1959 the urea clearance was 82 cc./min. A recent electrophoretic pattern of his plasma is shown (FIGURE 5).

Comment

The patient has been under observation for 4 years and has a history of almost 6 years of edema. The edema has been intermittent, although its degree has ranged widely, from slight pitting edema of the ankles to massive anasarca. During this period the kidney function has remained within normal limits. The patient has been in reasonably good health otherwise, and has shown no

evidence of systemic disease. His edema responds well to adrenal cortical steroids.

Case 6

A mulatto male, born in 1901 was seen in the University of Chicago Clinics for the first time in October 1958. He gave a history of ankle swelling of 6 weeks. One year prior to admission he had had intermittent, early morning aching in the proximal interphalangeal joints of both hands without swelling or redness. In addition to the dependent edema, he also had noted shortness of breath on climbing 3 flights of stairs. There was no history of other joint involvement, pleurisy, or allergy.

Physical Examination

The patient had a blood pressure of 160/110 mm. Hg, with a regular heart rate of 72/min. There were no rashes, the lymph nodes were not enlarged, and the spleen was not palpable. The patient had pitting edema of the extremities and of the sacrum and crepitus of the right knee.

Laboratory Data

The hemoglobin was 11.8 gm./100 ml.; the red cell count was 4.25; the sedimentation rate was 58 mm./hour; the urine always contained 4+ albumin, frequently with coarse and fine granular casts and occasionally with fatty casts with doubly refractile fat; there were 8 to 10 red blood cells per high-power field; 3 preparations for lupus were normal; the total cholesterol was 480 mg./100 ml. The plasma albumin was 1.5, 1.2, and 1.4 gm./100 ml.; the globulin was 3.9 and 3.3 gm./100 ml. The heart size appeared normal by X-ray examination. The electrocardiogram was normal. The patient improved with a low salt diet, prednisone, and bedrest.

Renal biopsy. Biopsy performed at the first admission shows 22 glomeruli in an area of unscarred cortex. The glomeruli have a diffuse thickening of the capillary loops with material that stains as a blood protein. Electron microscopy reveals an extrinsic membranous deposit with inclusions in the cytoplasm of the epithelial cells. The glomeruli are normally cellular, have normal capillary lumina, and there is no debris in Bowman's capsule. There is a slight increase in interstitial fluid and an occasional small focal infiltration with lymphocytes. Scattered hyaline casts are seen, and some tubular cells contain hemosiderin pigment.

Physical Examination, Second Admission

The patient was hospitalized again because of epigastric pain and black stools. An X-ray diagnosis of peptic ulcer and bronchopneumonia was made. These responded to medical management. Another hospitalization was made for fever, a rash, and abdominal cramps, presumably due to sensitivity to phenobarbital. Progressive swelling of the arms and legs and exertional dyspnea and orthopnea necessitated readmission. These complaints have responded indifferently to therapy, and a chronic anasarca has persisted to the time of this report. A number of experimental drugs for relief of this edema has been tried without effect.

Laboratory Data

The plasma protein has fallen as low as ½ gm. of albumin per 100 ml. with a globulin of 4.6 gm./100 ml. The cholesterol has remained high. Numerous preparations for L.E. cells have been negative. There has been a maximal proteinuria of 48 gm./day.

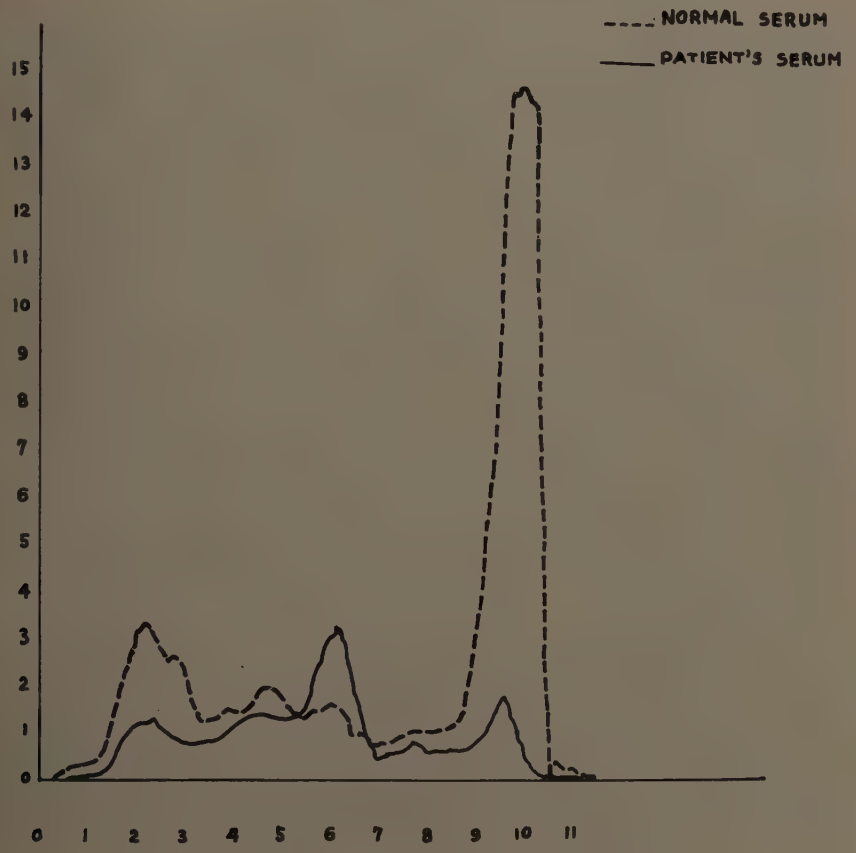

FIGURE 6. Electrophoresis of serum from patient 6.

In 1958, the urea clearance was 52 cc./min.; hemoglobin was 11 gm./100 ml. In 1959, the urea clearance was 56 cc./min. (square root formula), and hemoglobin was as low as 6.7 gm./100 ml. The reticulocyte response had been good during this time, with a high of 12.4 per cent. The serum calcium was observed at 7, and phosphorus at 5 mg./100 ml. The other serum electrolytes were normal. A glucose tolerance test gave a normal result. The electrophoretic pattern during one of the severe episodes of anasarca is shown in FIGURE 6.

A repeat renal biopsy on March 11, 1959, showed no progression of the lesion.

Comment

Except for anasarca, this patient shows a minimal systemic reaction associated with EMD of the glomerulus of the kidney. If these systemic findings are significant, then the most that can be found in support of a diagnosis of generalized collagen disease is the short history of joint pain and crepitus. The patient's response to steroids has been minimal.

On his initial admission, the patient responded well to salt restriction and prednisone. Since then, however, the drug has not reduced the degree of albuminuria. Despite his continued and rather massive albumin loss over a period of 2½ years, his renal function, as measured by urea clearance, has remained normal. There have been no L.E.-positive preparations. Basically, this appears to be a disorder of the connective tissue, with a minimal cellular reaction confined almost exclusively to the kidney.

Discussion

The glomerular EMD found in these cases is significant for several reasons. As a striking and conspicuous lesion seen in the biopsy series, it may have diagnostic importance. Furthermore, the morphology of the lesion provides a real clue to its pathogenesis. The one constant concomitant of the EMD has been the presence of albuminuria. In addition, the full nephrotic syndrome frequently is present at some point in the course of the disease.

The EMD seems to consist of variable amounts of protein material deposited along the outer surface of the basement membrane (FIGURE 2). The basement membrane, usually a uniformly hyaline structure, is less dense in this area, has less regular margins, and frequently may be shown to contain vacuoles (FIGURE 3). Occasionally it has droplets that stain as the main deposit, suggesting the movement of visible masses of material from the capillary lumen to the urinary space. These morphologic changes in the adjacent basement membrane, as well as the proteinuria, suggest that the EMD is formed by the deposition of some macromolecules derived from the blood. An alternative hypothesis is that some of the usually soluble molecules derived from the cells of the glomerulus have coagulated *in situ*.

The epithelial cell change is variable. In many instances the foot processes remain, but are separated by dense material; however, in other areas the foot processes have been separated from the basement membrane and focally distorted (FIGURE 2). The epithelial cell cytoplasm is vacuolated and contains droplets (FIGURE 7); however, this is a common nonspecific change that we have seen in almost every biopsied case with proteinuria. Although the change in permeability of the basement membrane is the most important functional disturbance associated with the EMD lesion, the EMD may be the consequence of this rather than the cause. In our series, the former is suggested by the fact that a number of cases show severe prolonged proteinuria in the presence of the nephrotic syndrome without having the EMD.

As presented under *Observations*, this lesion has been found in 6 cases that clinically presented with the nephrotic syndrome. In addition, 2 cases show evidence of systemic lupus erythematosus. The remaining 4 cases have no clearly diagnosable systemic disease but several show mild systemic complaints referable to the connective tissue.

Several problems are posed by these findings. First, although the lesion is found in 2 cases of systemic lupus erythematosus, is it a necessary part of the lupus syndrome? In our series, 7 other cases with lupus nephritis did not have this lesion. They showed a change most marked in the basement membrane and the endothelial cells.[9] Frequently, the basement membrane was 2 to 4 times its normal thickness (FIGURE 8) but remained hyaline with no changes

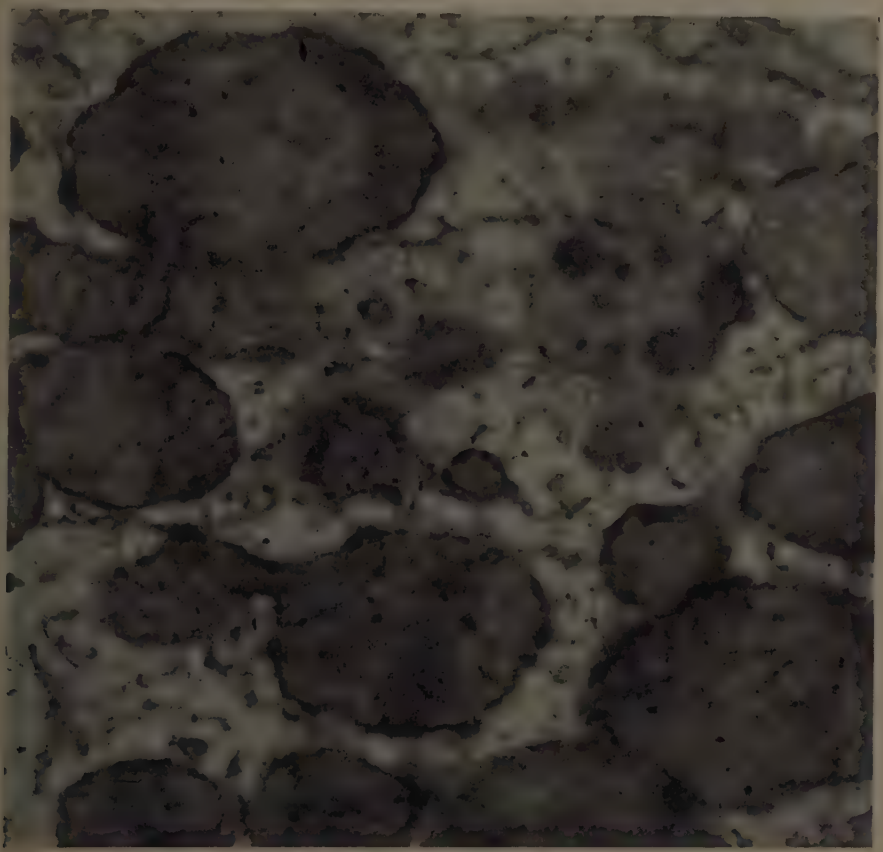

FIGURE 7. This glomerular epithelial cell contains many cytoplasmic droplets, some of which contain remnants of mitochondrial cristae. These droplets are frequently found with proteinuria. ×15,000.

of the type seen in EMD. Deposits described as fibrinoid were inside the basement membrane (FIGURE 9) and the endothelial cells, in the cases of systemic lupus erythematosus,[10] in contrast to the EMD, which was associated closely with the epithelial cells. The possibility must still be considered that the EMD is an early stage or a variant of the lupus nephritis lesion as this is now described.

The second possibility that must be considered is that this is a general phenomenon of collagen diseases affecting the kidney. On this point we may re-

FIGURE 8. The changes of lupus nephritis frequently include thickening of the basement membrane (BM), proliferative changes in the endothelial cell (END NUC), and hyaline thrombi (HY TH). ×4000.

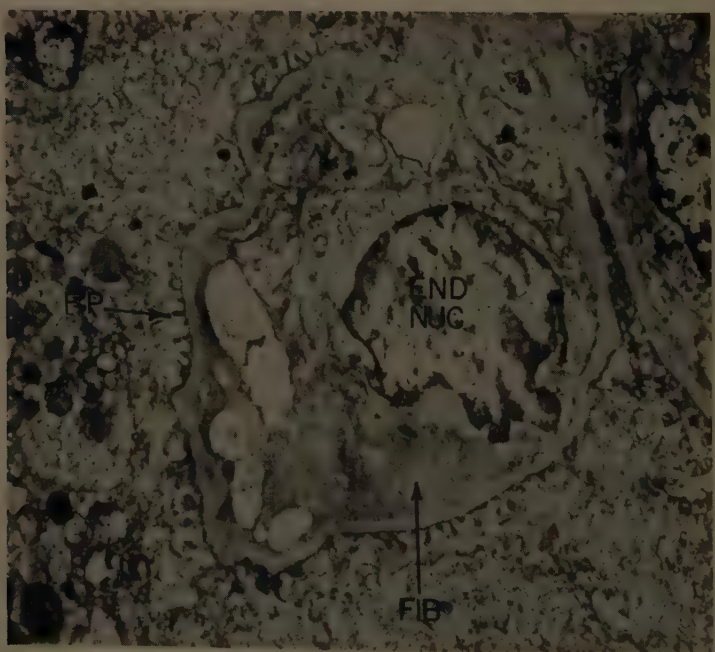

FIGURE 9. Dense osmophilic deposits (FIB) inside the basement membrane. Lupus nephritis. ×4000.

view examples of specific connective tissue diseases from our series. Renal changes of rheumatoid arthritis may be demonstrated where there is moderate proteinuria (FIGURE 10). In this disorder, the basement membrane of the glomerular capillary is thickened uniformly. There are areas of irregular density. Endothelial proliferative changes are less marked than those of lupus nephritis and are more focal. Acute rheumatic fever also frequently has

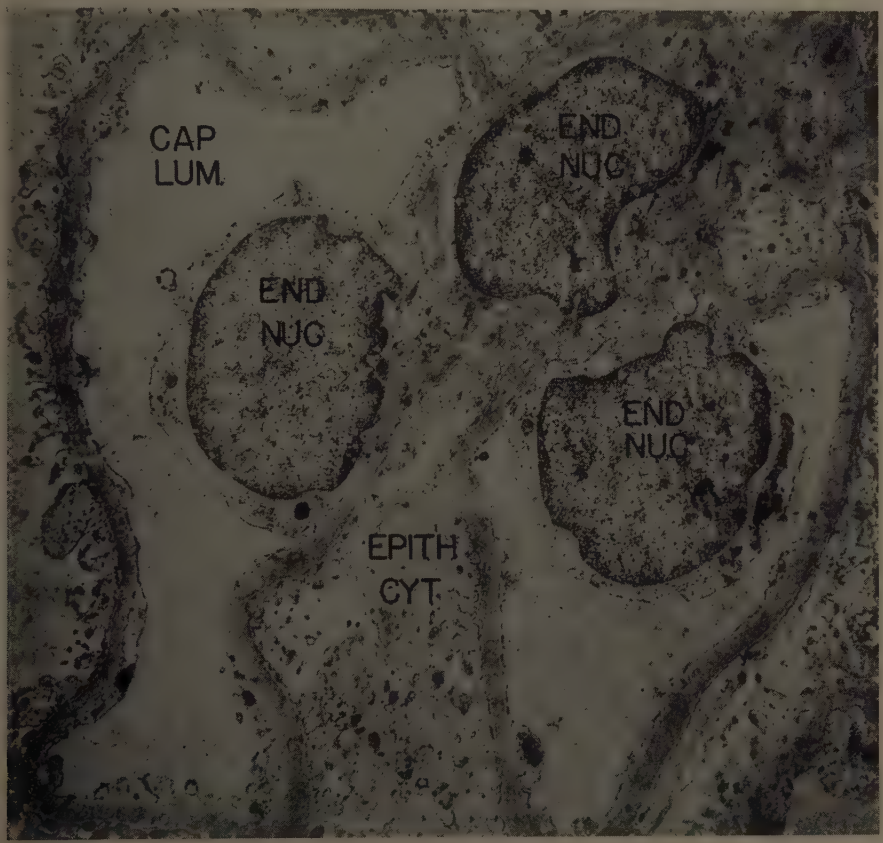

FIGURE 10. A mild focal endothelial cell proliferative glomerular change. Rheumatoid arthritis. The basement membrane is thickened and occasionally contains vacuoles. ×4000.

glomerular lesions. The most prominent change is a severe endothelial cell proliferation with irregular folding and thickening of the basement membrane, especially in the lobular stalk area (FIGURE 11). The lesions of the latter type may take on a variety of forms, depending on the degree of cellular exudate and epithelial cell proliferation. Chronic glomerulonephritis[10] frequently has few glomeruli, and much of the fine structure is obliterated (FIGURE 12). There is a conspicuous endothelial cell proliferation and fusing of the glomerular capillaries into a lobular pattern. In scarred areas, epithelial cell foot processes may be absent. Fibroepithelial crescents were rare in our material. In

areas of the most marked endothelial cell proliferation there are basement membrane changes, especially in the area of the lobular stalk. This last change, which appears to be nonspecific in type, may be produced by glomerular ischemia. Complete hyalinization of the glomerulus is produced by a peripheral extension of the basement membrane folding, with loss of cells. Secondary amyloidosis[11] has a pathognomonic appearance by electron microscopy. The material is fibrillar and contrasts with the hyaline basement mem-

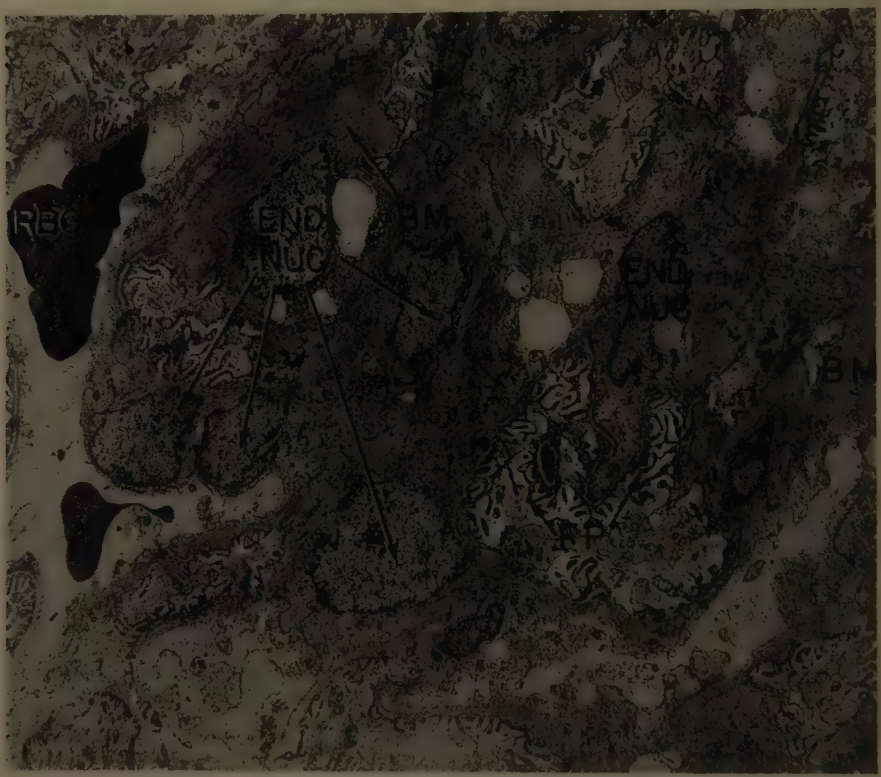

FIGURE 11. A partially fused lobule from a case of acute rheumatic fever, showing the preservation of the foot processes of the epithelial cells (FP).

brane (FIGURE 13). Larger accumulations extend into the capillary lumen and distort the endothelial cell by extending through the basement membrane into the epithelial cell. In none of these connective tissue diseases showing renal manifestations is there evidence of a change of the extrinsic membranous type.

The relationship between the EMD and the poorly defined clinical concept, lipid nephrosis, also must be considered. In each situation there are conspicuous epithelial cytoplasmic changes. Farquhar[4] and Movat and McGregor[5] have described electron photomicrographs of a similar change that they interpreted as a form of adult lipid nephrosis. Since lipid nephrosis even in childhood is a poorly defined clinical entity without a known etiology or pathogen-

esis, it is difficult to exclude from these considerations. The one definite diagnostic sign of lipid nephrosis is fusion of the glomerular foot processes;[12] this has not been a consistent finding in our series of cases with EMD.

Obviously, our present data do not resolve the problem of the nature of the EMD and its associated clinical findings. We believe, however, that the size

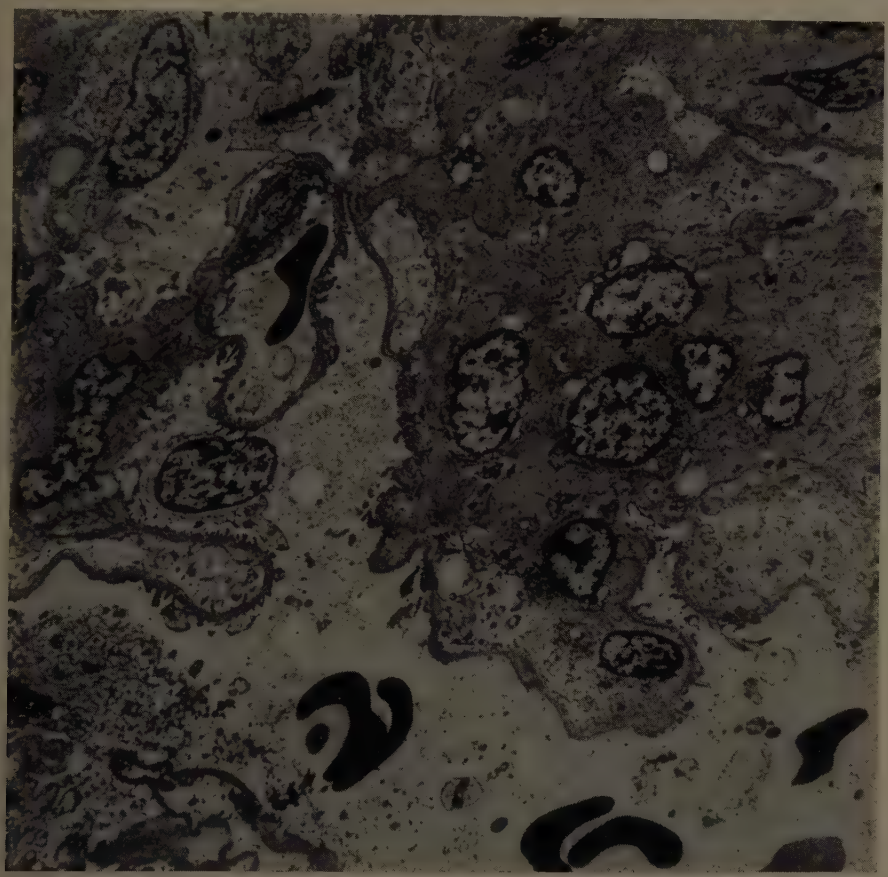

FIGURE 12. Chronic glomerulonephritis. Extensive distortion of the glomerular structures is shown, with folding and thickening of the basement membrane in areas of endothelia proliferation. ×3000.

of the sample is sufficiently large to draw a tentative conclusion on epidemiological grounds, that EMD represents a unique entity belonging to the connective tissue group of diseases. The evidence of its association with this group of diseases is found first of all in its morphologic nature. Thus the EMD seems to involve primarily the ground substance and secondarily the cells. Strong clinical evidence of a syndrome closely related to lupus is found in two of the four cases that have a variety of findings suggestive of the connective tissue diseases.

Evidence that this may be a unique lesion is taken from the fact that a variety of related syndromes has been studied without finding this lesion. It may be pointed out that sampling errors have been minimized in this study by the fact that the lesion is so diffuse and uniformly distributed that examination of even a portion of a glomerulus reveals its presence. As a working hypothesis,

FIGURE 13. Secondary amyloidosis (AM) involving the glomerulus has a distinctive appearance. It is frequently between the basement membrane and the endothelial cell. Larger accumulations occlude the capillary lumen (CAP LUM) and extend through the basement membrane to the epithelial cell (EP CYT). ×4500.

we prefer the interpretation that the EMD and its associated clinical findings comprise a member of the connective tissue group of diseases that may even be a variant of systemic lupus.

References

1. JONES, D. B. 1953. Glomerulonephritis. Am. J. Pathol. **29:** 33–51.
2. JONES, D. B. 1957. The kidney. Inflammatory and vascular disease of the glomerulus. *In* Analytical Pathology. : 161–217. R. C. Mellors, Ed. McGraw-Hill. New York, N. Y.

3. Churg, J. & E. Grishman. 1959. Subacute glomerulonephritis. Am. J. Pathol. **35**(1): 25–45.
4. Farquhar, M. 1958. *In* Proceedings of the 10th annual conference on The Nephrotic Syndrome. : 2–6, 8–19, 21, 23, 24, 25, 26, 90, 92. J. Metcoff, Ed. New York, N. Y.
5. Movat, H. Z. & D. D. McGregor. 1959. The fine structure of the glomerulus in membranous glomerulonephritis (lipoid nephrosis) in adults. Am. J. Clin. Pathol. **32**(2): 109–127.
6. Kark, R. M. & R. C. Muehrcke. 1954. Biopsy of the kidney in the prone position. Lancet. **1:** 1047.
7. Palade, G. E. 1952. A study of fixation for electron microscopy. J. Exptl. Med. **95:** 285.
8. Hargraves, M. M. 1949. Proc. Staff Meet. Mayo Clinic. **24:** 234.
9. Arnold, J. D. & B. Spargo. 1959. Clinical use of the percutaneous renal biopsy. Circulation. **19**(4): 609–621.
10. Vernier, R. L., M. G. Farquhar, J. G. Brunson & R. A. Good. 1958. Chronic renal disease in children. Correlation of clinical findings with morphologic characteristics seen by light and electron microscopy. A.M.A. J. Diseases of Children. **96:** 306–343.
11. Geer, J. C., J. P. Strong, H. C. McGill, Jr. & I. Muslow. 1958. Electron microscopic observations on the localization of amyloid in the kidney in secondary amyloidosis. Lab. Invest. **7**(6): 554–565.
12. Farquhar, M. G., R. L. Vernier & R. A. Good. 1957. Studies on familial nephrosis. II. Glomerular changes observed with the electron microscope. Am. J. Pathol. **33**(4): 791–817.

REACTIONS TO HOMOLOGOUS AND HETEROLOGOUS FIBRIN IMPLANTS IN EXPERIMENTAL ANIMALS

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The eosinophilic necrotic material that is so conspicuous a feature in the center of a rheumatoid nodule consists of fragments of collagen in various stages of disintegration and embedded in a noncollagenous matrix. The latter is composed largely of fibrin and other exuded plasma proteins, as revealed by staining with fluorescent antibodies (Gitlin *et al.*, 1957; Banerjee, 1958), and is digested readily by trypsin (Ziff *et al.*, 1953; More and Movat, 1957). In view of this ready digestibility by trypsin and the presence of trypsinlike enzymes in the tissue fluids, it is difficult to explain the persistence of these plasma proteins within the nodule. In the hope that some light would be thrown on this problem a study was undertaken of fibrin fragments implanted subcutaneously in experimental animals.

Fibrin was prepared aseptically from blood taken into citrate dextrose solution by adding either bovine thrombin, 25 U./100 ml., or calcium chloride, the red cells having been removed previously by centrifugation. After the clot had retracted fully, it was cut into fragments, and excess fluid was removed by squeezing with sterile gauze. Fibrin was obtained in this way from rats, guinea pigs, sheep, cows, and humans, and fragments weighing 200 to 250 mg. were implanted subcutaneously into rabbits and, occasionally, into rats and guinea pigs. Implants were removed at intervals between 4 and 56 days after implantation, fixed in formol-saline, and studied histologically. In some animals the reaction to repeated implantation also was studied. Samples of fibrin were tested for sterility before and after implantation.

The results as illustrated in FIGURES 1 to 6 show a striking difference between the homologous and the heterologous implants. In rabbits a homologous implant at 7 days (FIGURE 1) is penetrated diffusely by plump fibroblasts and capillaries; at 14 days (FIGURE 2) the processes of the fibroblasts form a diffuse network with consequent fragmentation of the fibrin, while at 21 days (FIGURE 3) the implant virtually is replaced by a fibrocellular scar so small that it is often difficult to find with the naked eye. The reaction to a heterologous implant differs little from that to a homologous implant for the first 4 days, but subsequently the invading cells undergo a rapid necrosis and their nuclear debris may form a conspicuous zone at its margin (FIGURE 4). The host tissue surrounding the implant becomes the site of a considerable proliferation of fibroblasts and histiocytes that, by about the twenty-fifth day, have arranged themselves with their long axes at right angles to the surface of the implant, thus forming a palisade strongly reminiscent of that seen characteristically around the necrotic zone of a rheumatoid nodule (FIGURES 5 and 6). More peripherally, in the loose connective tissue, a considerable new formation of small vessels occurs, not diffusely but in focal aggregations associated with a variable degree of perivascular cellular infiltration. This gives the appearance

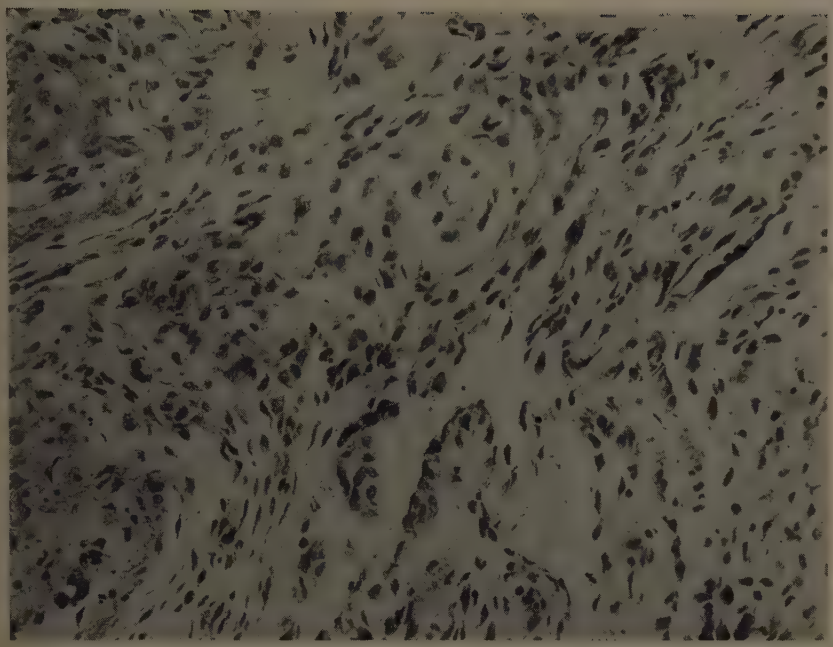

FIGURE 1. Rabbit fibrin implanted in a rabbit. After 7 days there is extensive invasion of the implant by fibroblasts and capillaries. Hematoxylin and eosin; ×140.

FIGURE 2. An implant similar to that shown in FIGURE 1 removed after 14 days. The fibroblastic infiltration has penetrated to the center of the implant, which is separated into small fragments by the meshwork of cell processes. Hematoxylin and eosin; ×140.

of multiple vascular granulomata in the vicinity of the implant (FIGURES 7 and 8). This cellular infiltration consists chiefly of lymphocytes and plasma cells, but has small numbers of polymorphs in the early stages. Here, too, the resemblance between these granulomata and those found typically in the periphery of rheumatoid nodules is remarkable (FIGURE 9).

Since the physical and chemical differences between fibrins of different mammalian species are small, fibrins probably are not responsible for the observed differences in response. On the other hand, the known immunological differences suggest strongly that the peculiarities of the response to the heterologous

FIGURE 3. A homologous fibrin implant in a rabbit, removed after 21 days. The implant virtually has been replaced by a moderately vascular fibrocellular scar. Hematoxylin and eosin; ×112.

implants are the expression of an immunological reaction to a mass of foreign protein. This interpretation is supported by the following observations:

(1) The differences between the two types of implant are not immediately apparent. Presumably, the time interval is that required for the immune reactions of the host to become manifest.

(2) Circulating antibody to bovine fibrinogen was found in the serum of a rabbit 50 days after the animal had received an implant of bovine fibrin. This was demonstrated readily by the Ouchterlony plate method. A positive Arthus reaction also was given by the same animal when injected intracutaneously with bovine fibrinogen. Since heterologous fibrin is strongly antigenic, there can be little doubt that similar antibodies to the other foreign fibrins would be found if sought for in the recipients of the foreign implants.

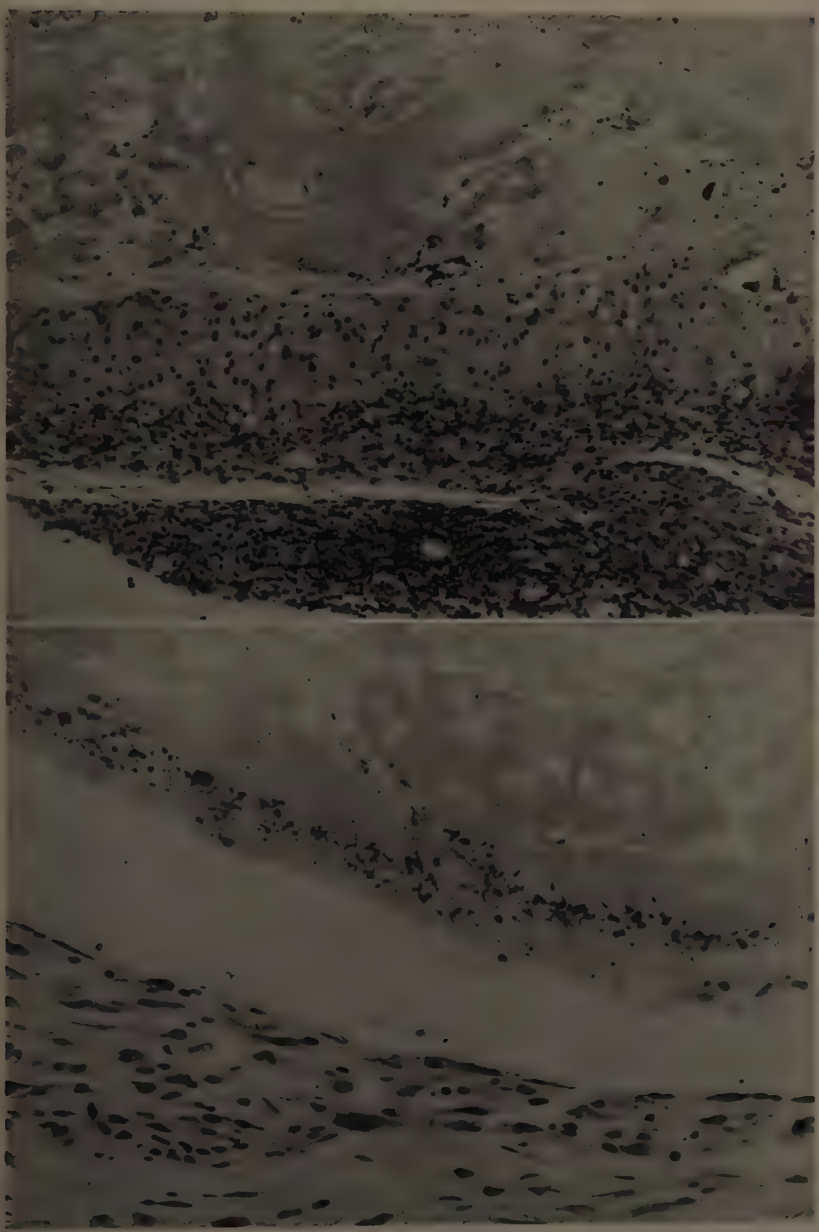

FIGURE 4. (*Top*) A heterologous implant of sheep fibrin into a rabbit, removed after 28 days. The cells that have invaded the fibrin have undergone necrosis, and their pyknotic and fragmented nuclei are scattered through its peripheral zone. The cellular palisade is conspicuous. Hematoxylin and eosin; ×112. (*Bottom*) Bovine fibrin removed 25 days after implantation in a rabbit. The marginal zone of the implant is heavily infiltrated with the nuclear debris of necrotic cells. Living cells are conspicuously absent from the implant, but form a well-defined capsule around it. The gap between them is a fixation artifact. Hematoxylin and eosin; ×210.

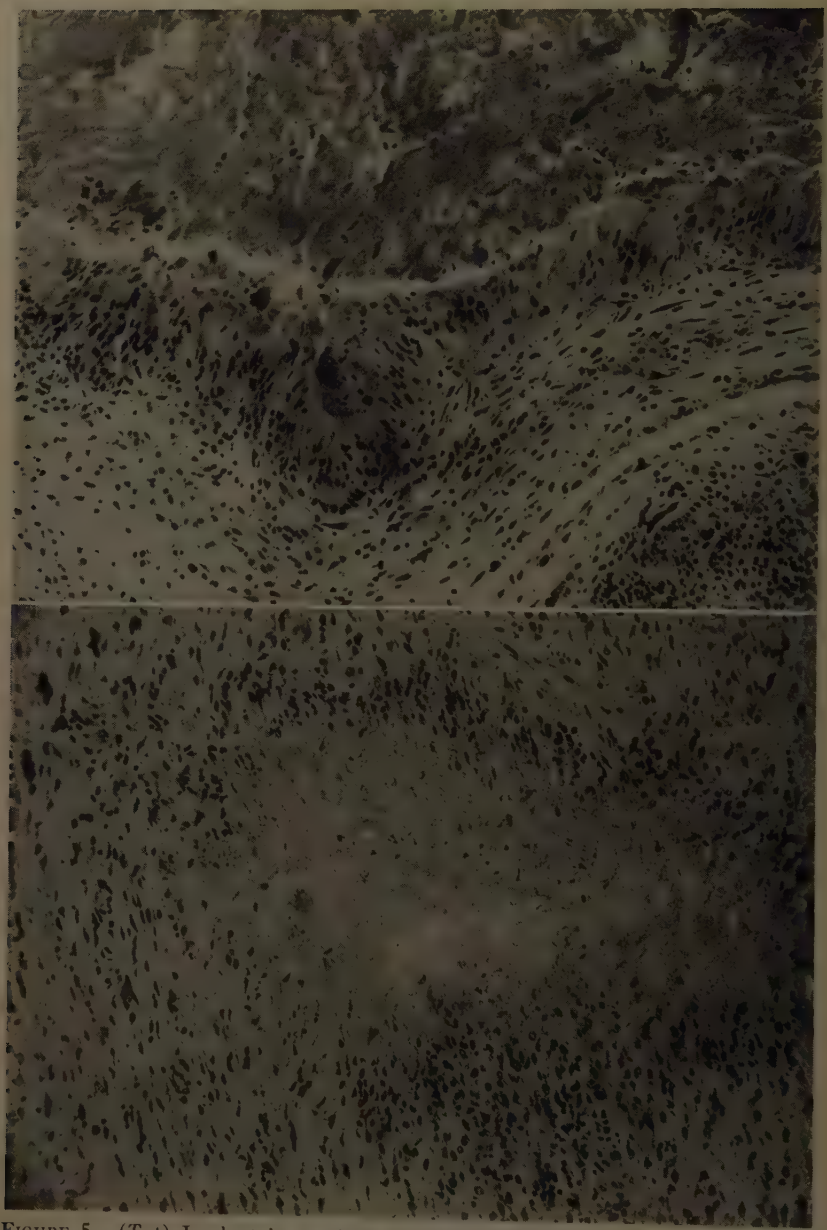

FIGURE 5. (*Top*) Implant in a rabbit of human fibrin removed after 28 days. This shows a well-marked palisade and the failure of cellular invasion. Hematoxylin and eosin; ×140. (*Bottom*) A rheumatoid nodule showing the typical cellular palisade. Hematoxylin and eosin; ×112.

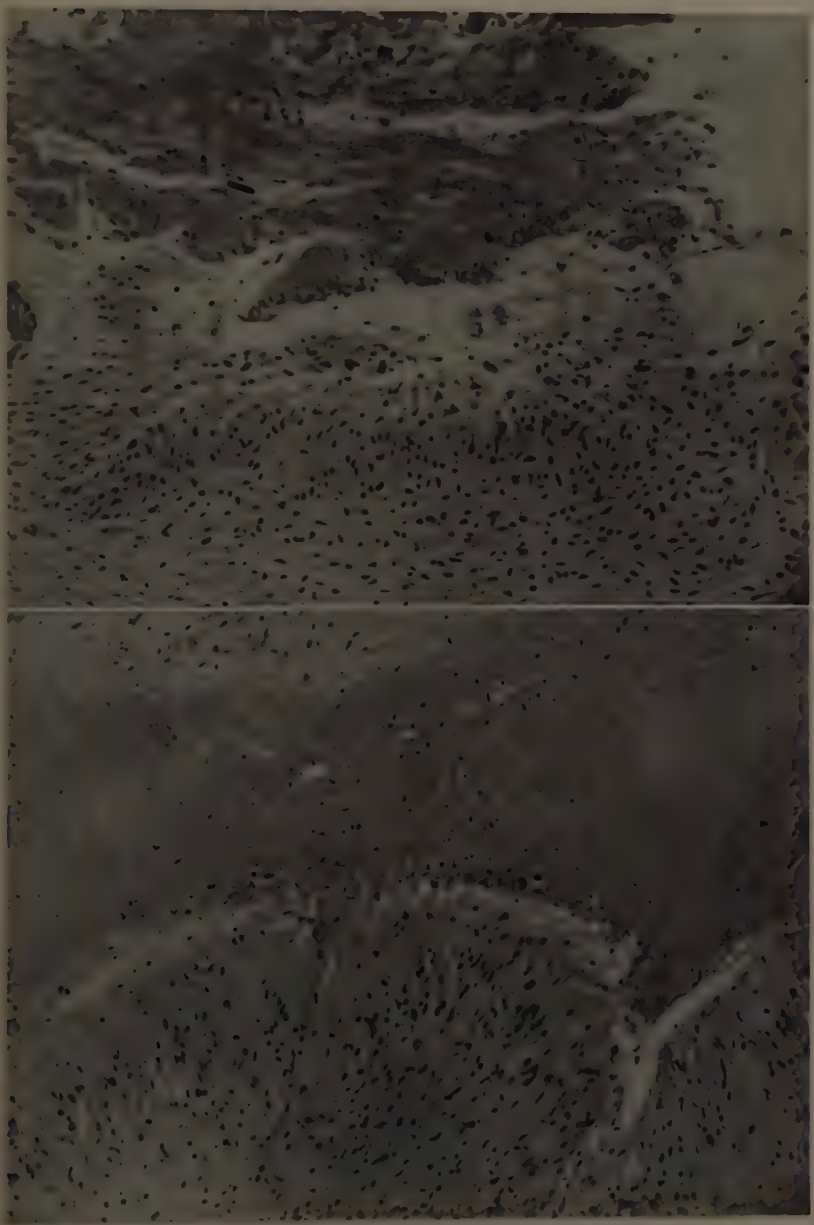

FIGURE 6. (*Top*) Implant of sheep fibrin in a rabbit, removed after 14 days. The palisade formation is already well developed. Hematoxylin and eosin; ×112. (*Bottom*) Another rheumatoid nodule, showing the close resemblance of its palisade to that formed around a heterologous implant. Hematoxylin and eosin; ×112.

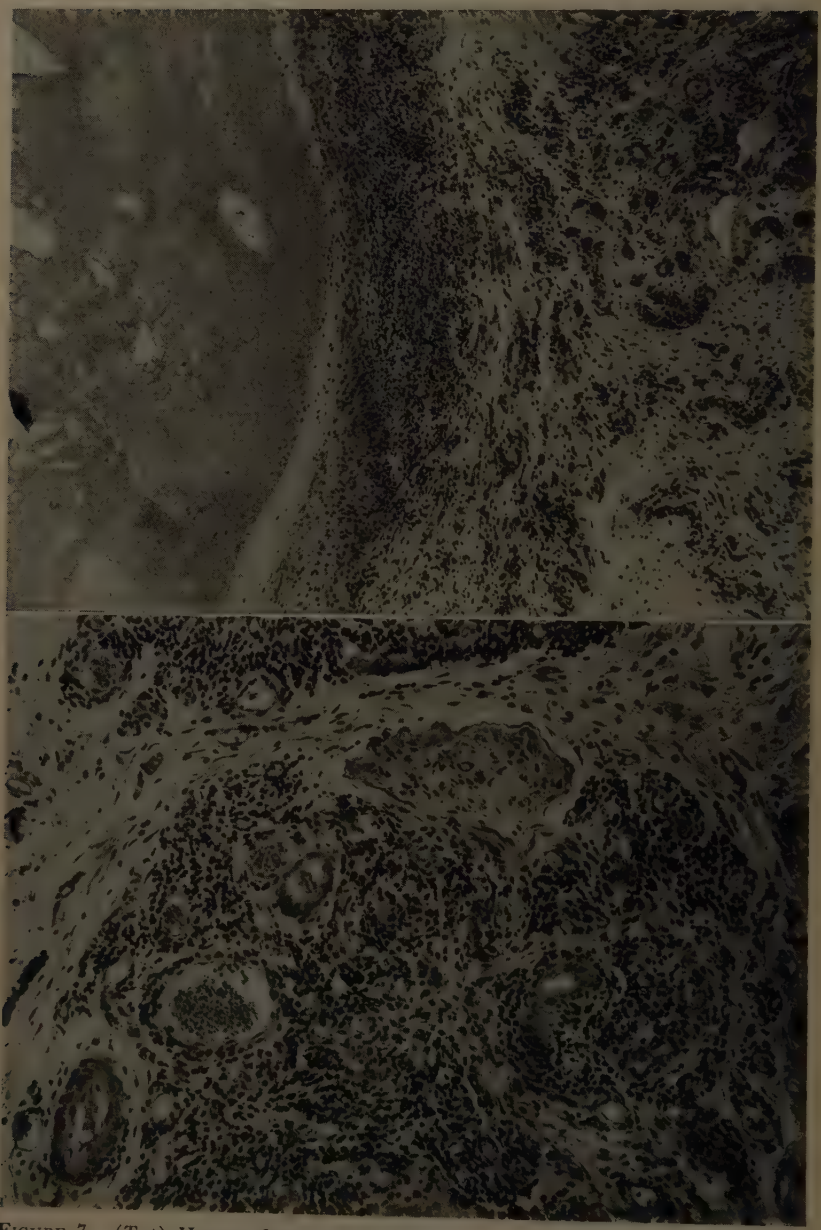

FIGURE 7. (*Top*) Human fibrin implant removed from a rabbit after 25 days. The developing vascular granulomata are seen in the connective tissue in the vicinity. Hematoxylin and eosin; ×60. (*Bottom*) Vicinity of a bovine fibrin implant removed from a rabbit after 4 days. It had been implanted 4 months after a previous implant. Note the early development of the granulomata. Hematoxylin and eosin; ×112.

(3) A large number of plasma cells is present both in the perivascular infiltrations and, more diffusely, in the loose areolar tissue adjacent to the implants.

(4) There is a suggestion of an accelerated reaction to reimplantation of heterologous fibrin.

(5) Acute necrotizing arteritis is found in the area of such accelerated reactions (FIGURE 10).

Thus the experimental results show that, when fibrin and its *in vivo* environment are foreign to each other, there is a breakdown of the usual mechanism of its absorption. The histological appearance suggests that fibrin, after a few

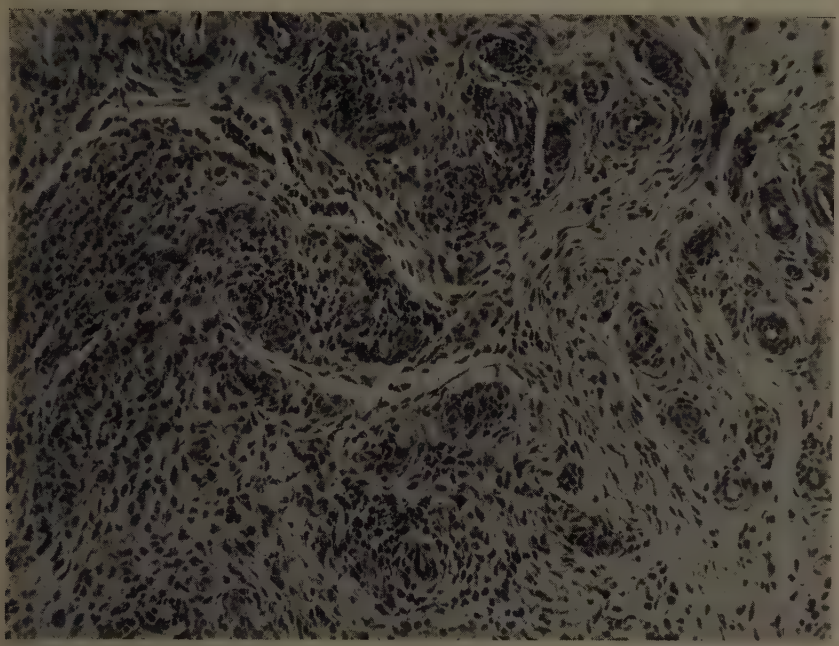

FIGURE 8. The vicinity of a rheumatoid nodule, showing the granulomatous reaction in the adjacent connective tissue. Hematoxylin and eosin; ×112.

days' sojourn in a heterospecific host, is a medium unsuitable for the survival of invading host cells. Moreover, this unsuitability apparently is a manifestation of an immune reaction of the host to the foreign protein of the implant. No satisfactory explanation, however, is yet available to account for the apparent resistance of the implant to the proteolytic enzymes of the tissue fluids. Whether this resistance is due to a lack of penetration of these enzymes through the palisade or to the deposition of some specific protective material within the implant remains to be resolved.

The chief interest of these experiments, however, lies in the remarkable similarity between the reaction to the heterologous implants and the subcutaneous nodule of rheumatoid arthritis. An outstanding difference is the presence within the so-called fibrinoid center of the nodule of fragments of collagen in various stages of disintegration. Any specific stain for collagen

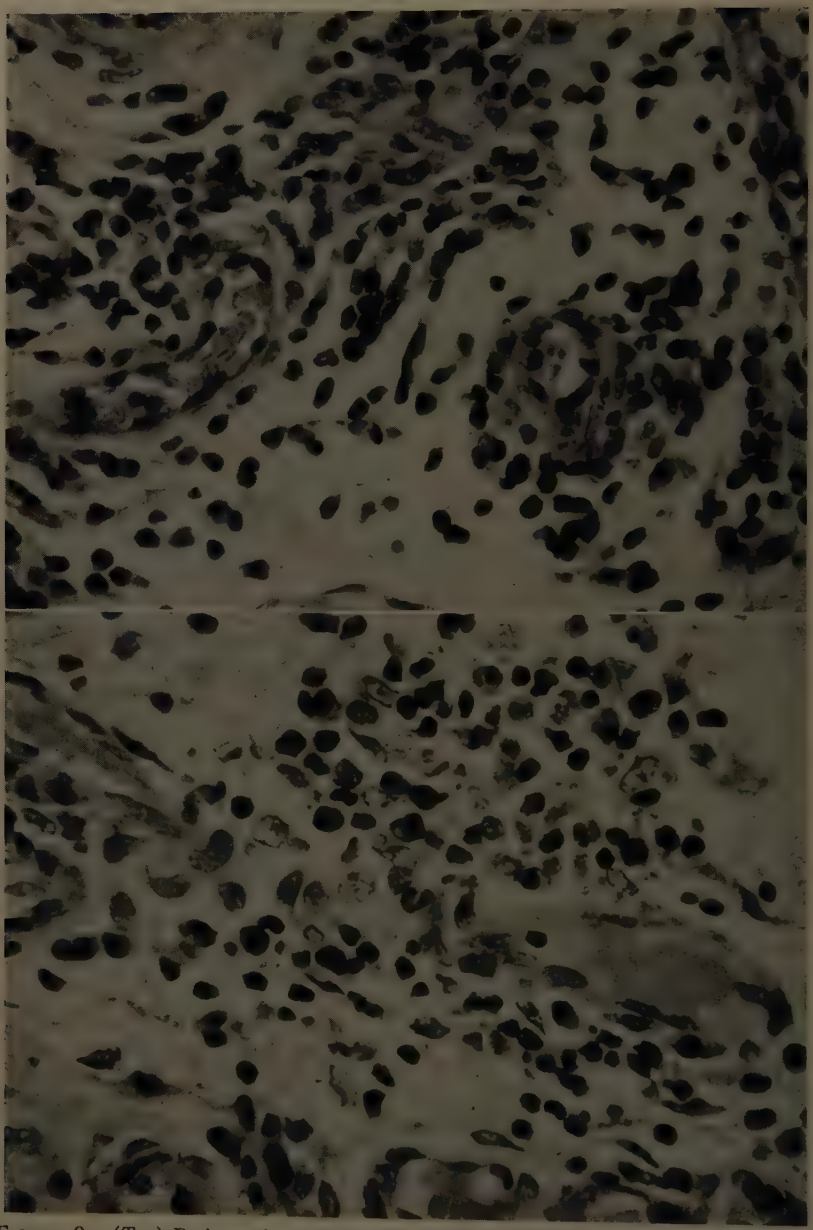

FIGURE 9. (*Top*) Perivascular cellular infiltration in a rabbit, in the vicinity of an implant of human fibrin after 14 days. The majority of the infiltrating cells belong to the plasma cell series. Hematoxylin and eosin; ×350. (*Bottom*) Perivascular cellular infiltration in the vicinity of a rheumatoid nodule. Here, too, the majority of the infiltrating cells are plasma cells. Hematoxylin and eosin; ×350.

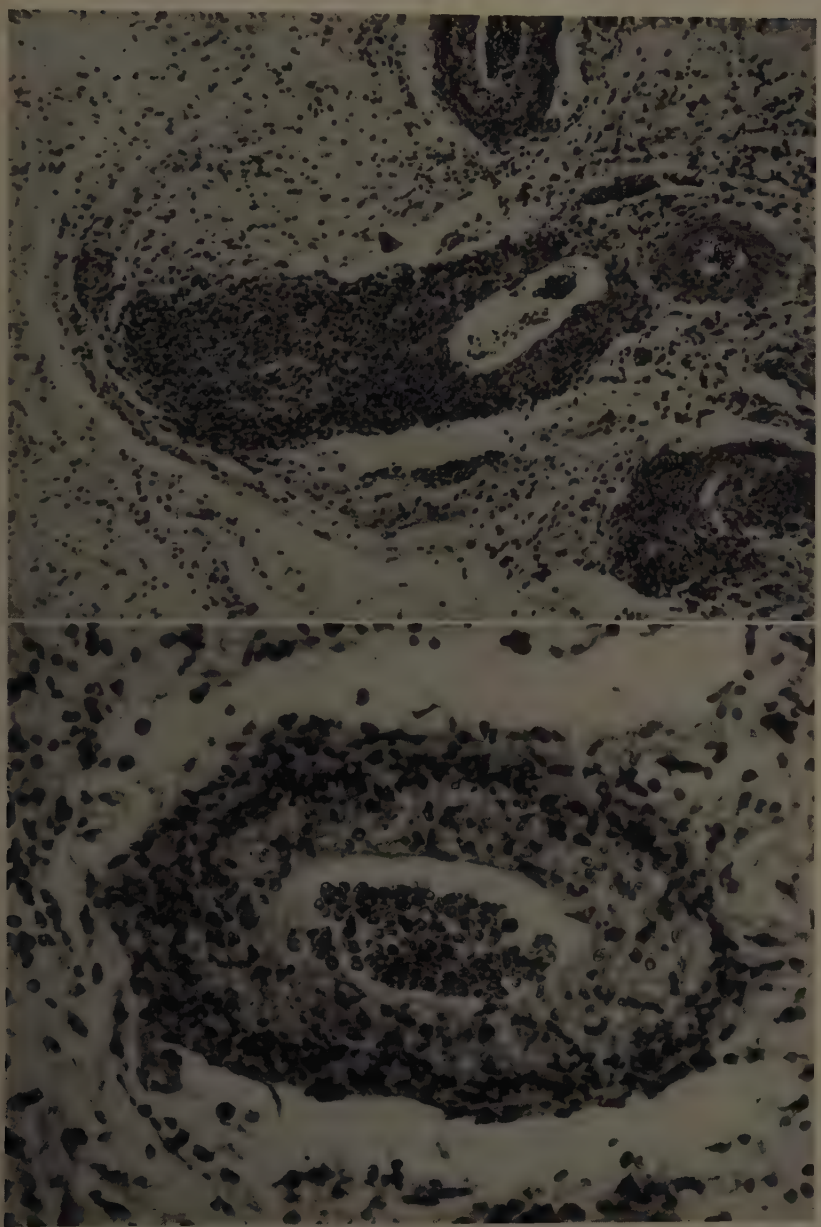

FIGURE 10. (*Top*) Acute necrotizing arteritis in the subcutaneous tissue of a rabbit 4 days after it had received an implant of bovine fibrin. The rabbit had received a similar implant 4 months before. Hematoxylin and eosin; ×112. (*Bottom*) One of the affected vessels in FIGURE 10, *top*. Hematoxylin and eosin; ×350.

therefore readily differentiates the two lesions. Nevertheless, the resemblance still is sufficiently close to arouse the suspicion that similar mechanisms are involved in their pathogenesis. We have noted that with the heterologous fibrin implant the mechanism is probably immunological and, although with the rheumatoid nodule an immunological pathogenesis still is almost entirely speculative, the role of autoimmunological reactions in the pathogenesis of rheumatoid arthritis by no means can be excluded. The Rose-Waaler test appears to many to be an example of such an autoimmune reaction because of the appearance in the blood of a macroglobulin capable of reacting specifically with the patient's own slightly altered gamma globulin. The presence of both these proteins within the substance of the nodule constitutes a noteworthy parallel to the heterologous fibrin implant in which, presumably, antigen and specific antibody also exist together. The strong correlation found in rheumatoid arthritics between a positive Rose-Waaler test and the presence of rheumatoid nodules could be interpreted as supporting the hypothesis of an immunological pathogenesis for the nodule, but thus far there is no other evidence of any causal relationship between them.

References

Banerjee, S. K. 1958. Ph.D. Thesis. Univ. London. London, England.
Gitlin, D., J. M. Craig & C. A. Janeway. 1957. Am. J. Pathol. **33:** 55.
More, R. H. & H. Z. Movat. 1957. Am. J. Clin. Pathol. **28:** 331.
Ziff, M., T. Kantor, E. Bien & A. Smith. 1953. J. Clin. Invest. **32:** 1253.

Discussion

Gustave J. Dammin (*Peter Bent Brigham Hospital, Boston, Mass.*): With the stains used in your sections, it appeared that, at the later time intervals, the center of the nodule did not stain as intensely. Has it been possible to quantitate an alteration in the heterologous fibrin at the center of the nodule?

Glynn: This may be possible when we apply fluorescent methods to the study of the nodule.

Bernard M. Wagner (*University of Washington, Seattle, Wash.*): The experimental production of fibrinoid by hypersensitivity mechanisms has shown that the material is largely fibrin. This is decidedly different from the studies of fibrinoid as noted in connective tissue diseases. A recent study in our laboratory of a rheumatoid nodule from a child with agammaglobulinemia revealed the presence of fibrinoid largely composed of collagen fiber material, acid mucopolysaccharides, and protein. Gamma globulin and fibrin were not localized in this fibrin by immunofluorescent techniques. This again points up the difficulty in relating experimental connective tissue diseases to human material.

PARENCHYMAL FIBROGENESIS: THE LIVER*

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Fibrosis in parenchymal organs is a reaction to injury which, particularly if progressive, interferes with the function of the parenchymal elements, as exemplified by chronic nephritis and hepatic cirrhosis. Fibrosis of the liver was investigated in this laboratory by fine structural and chemical techniques. This work was in continuation of studies of the distribution of connective tissue septa in relation to nodules, begun mainly by three-dimensional reconstruction in cooperation with Hans Elias.[1]

Sites of Hepatic Fibrogenesis

Fiber accumulation in the liver can result from aggregation of pre-existing connective tissue; for instance, following collapse after necrosis of the parenchymal cells or from new formation of fibers. Both processes are readily differentiated in typical cases by silver impregnation of the reticulum fibers. Fiber formation in the liver takes place in three locations: around liver cell plates, around proliferating ductules, and in the portal tracts. Each type shows common and different characteristics.[2]

Perihepatic cellular fibrogenesis. In the normal hepatic parenchyma a fine layer of amorphous periodic acid–Schiff (paS) positive material lines the liver cells and represents the sinusoidal wall, best seen in edema, when it is separated from the liver cell plates. The layer gives a histochemical reaction for acid and neutral mucopolysaccharides. In routine silver impregnations it appears to consist of membranes. However, if sections less than 1 μ thick are studied, individual, fairly widely spaced fibers are noted in this layer, usually represented as points because of cross-sectioning (FIGURE 1*A*). Under the electron microscope a continuous basement membrane similar to that seen around glandular structures is absent, and fine fibrils and bundles of fibrils with a periodicity of approximately 640 Å are found in the tissue spaces, frequently between the microvillous projections of the liver cells (FIGURE 2). In the presence of uncomplicated fatty metamorphosis or in regeneration of any kind, this connective tissue framework within the parenchyma appears rarefied because the connective tissue fibers are much farther apart. In contrast, if liver cell damage is present, the intralobular connective tissue is increased. This is seen in fatty metamorphosis with liver cell injury in alcoholic persons, particularly if Mallory's hyalin is present, and in prolonged viral hepatitis.

Small areas of collapse after liver cell loss are readily recognized by an irregular network of reticulum fibers replacing the liver cells (FIGURE 3*A*). In addition, around damaged liver cells a thickened cover of connective tissue is noted. For this, apparently, two processes are responsible.[3] One represents a

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duplication of the pre-existing framework, apparently after loss of entire liver cell plates and flattened aggregations of the framework supporting two neighboring plates (FIGURE 1*C*). This process is frequently associated with collagen staining in the duplicated fibers, suggesting new formation of fibers stimulated

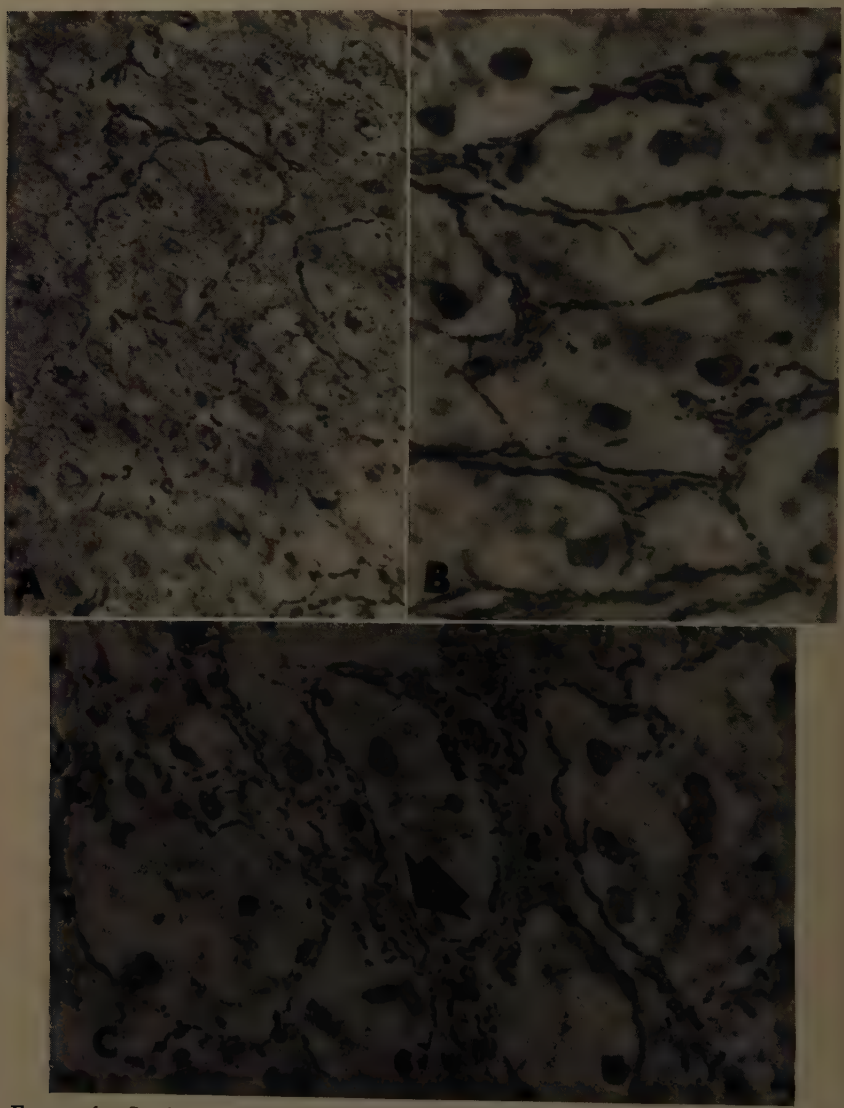

FIGURE 1. Sections of human liver biopsy specimens less than 1 μ thick. Silver impregnation of reticulum according to the method of Gomori. (*A*) Normal. The sinusoidal wall exhibits single reticulum fibers, most of them cut across and thus represented by single points, only in places forming lines. (*B*) Fatty metamorphosis with liver-cell damage in alcoholic. Increase of single fibers surrounding liver cells frequently merging to continuous membranes. (*C*) Viral hepatitis. Duplication of reticulum framework where liver-cell plates have disappeared (*arrow*) in flat collapse. ×630.

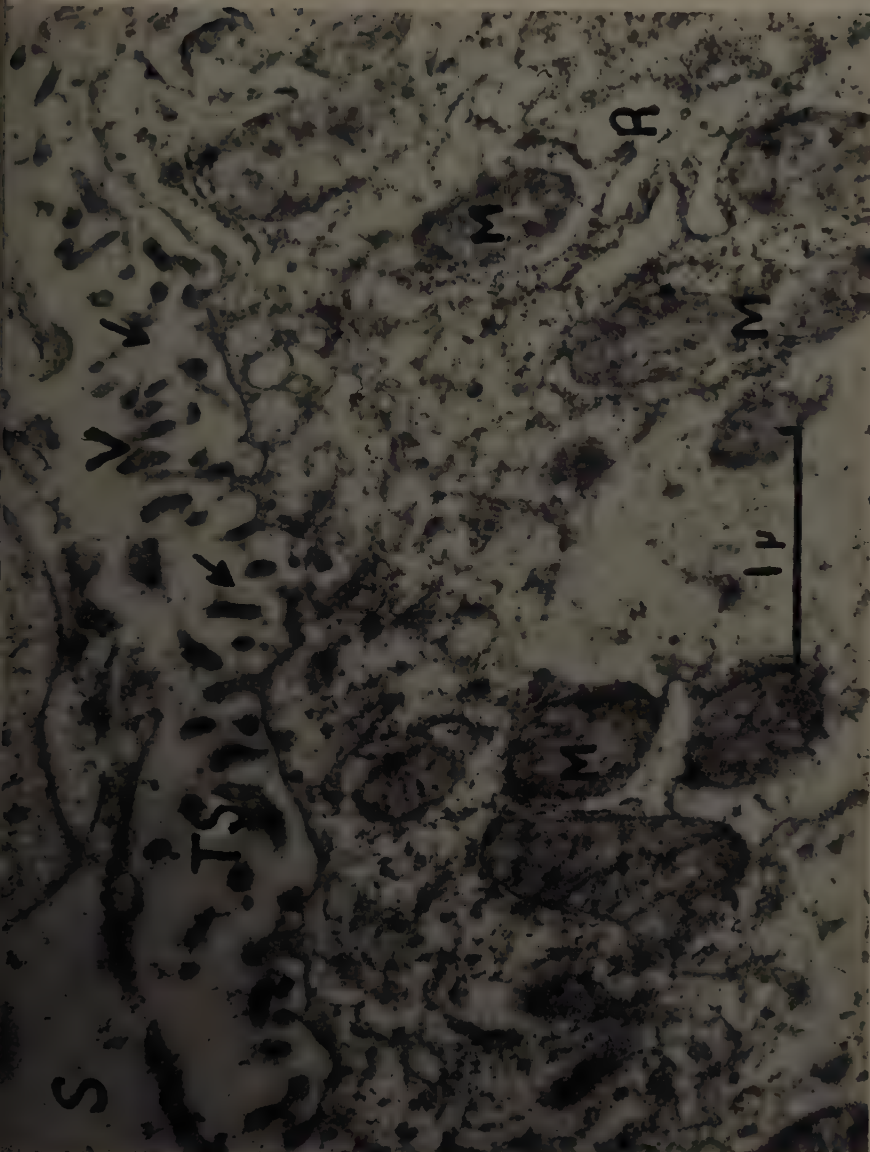

FIGURE 2. Electron photomicrograph of normal rat liver cell showing mitochondria (M) containing cristae, and endoplasmic reticulum (R) surrounded by numerous Palade granules. The cell border sends many projections or microvilli (V) into the tissue space (TS) separated from the sinusoid (S) by a thin endothelial layer. Between microvilli occasional single fibers are faintly seen (*arrows*). Osmium-fixed methacrylate-embedded. ×31,000.

by collapse. This suggestion is supported by chemical analysis.[4] This flat microcollapse also is readily apparent in electron-microscopy studies, in which thickened bundles are found in areas where liver cells had been. The other process is the increase in number of single fibers around the damaged and sometimes significantly swollen liver cells, so that in thin sections approximated fibers seem to merge, forming a continuous basement membrane (FIGURE 1*B*). The electron microscope reveals an increase of collagenous fibrils with characteristic periodicity (FIGURE 4) around such damaged liver cells. The fibers are arranged in thicker bundles up to 2 μ in diameter. Stains with paS show

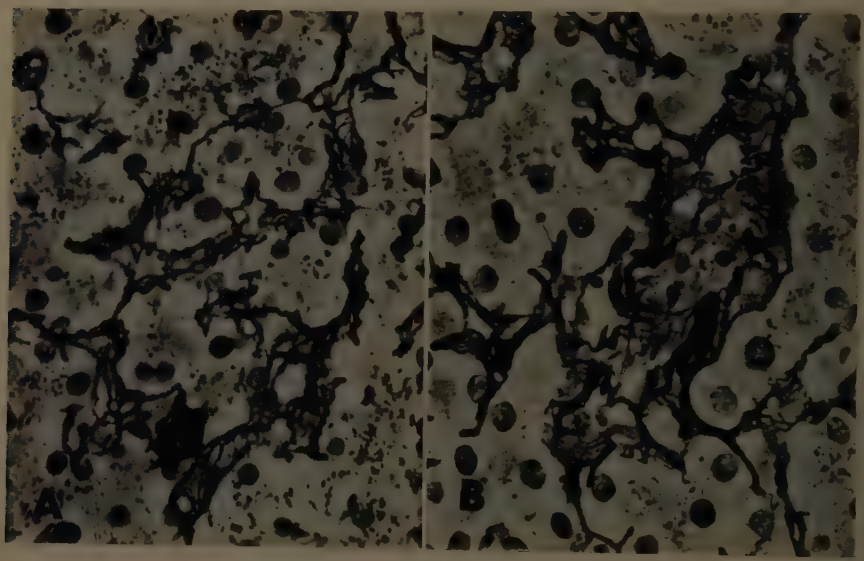

FIGURE 3. Liver biopsy specimen of viral hepatitis. Five μ-thick section. Silver impregnation according to the method of Gomori. (*A*) Irregular network of reticulum fibers resulting from loss of single or few cells (*arrow*) in microcollapse. (*B*) Increased connective tissue around proliferated bile ductules. ×400.

a thickened layer bordering the liver cells in the presence of liver cell damage, reflecting amorphous ground substances as well as fibers.

Whereas the Kupffer's cells normally have a distinctly paS-positive cytoplasm (even after digestion with diastase) with few strongly positive granules, in hepatocellular injury their increased cytoplasm appears crowded with paS-positive granules of various sizes. Cells of the cytological character of fibroblasts are seen rarely, if at all, in the vicinity of thickened fiber bundles, raising the question of the role of the Kupffer's cells in this fibrogenesis. The Kupffer's cells obviously are activated and mobilized. This and the histochemical similarity between their cytoplasmic granules and the thickened sinusoidal membrane, possibly ground substance, suggest that some of the granules are precursors of the matrix, as has been assumed for the fibroblasts elsewhere in connective tissue.[5] However, other histochemical reactions of the paS-positive

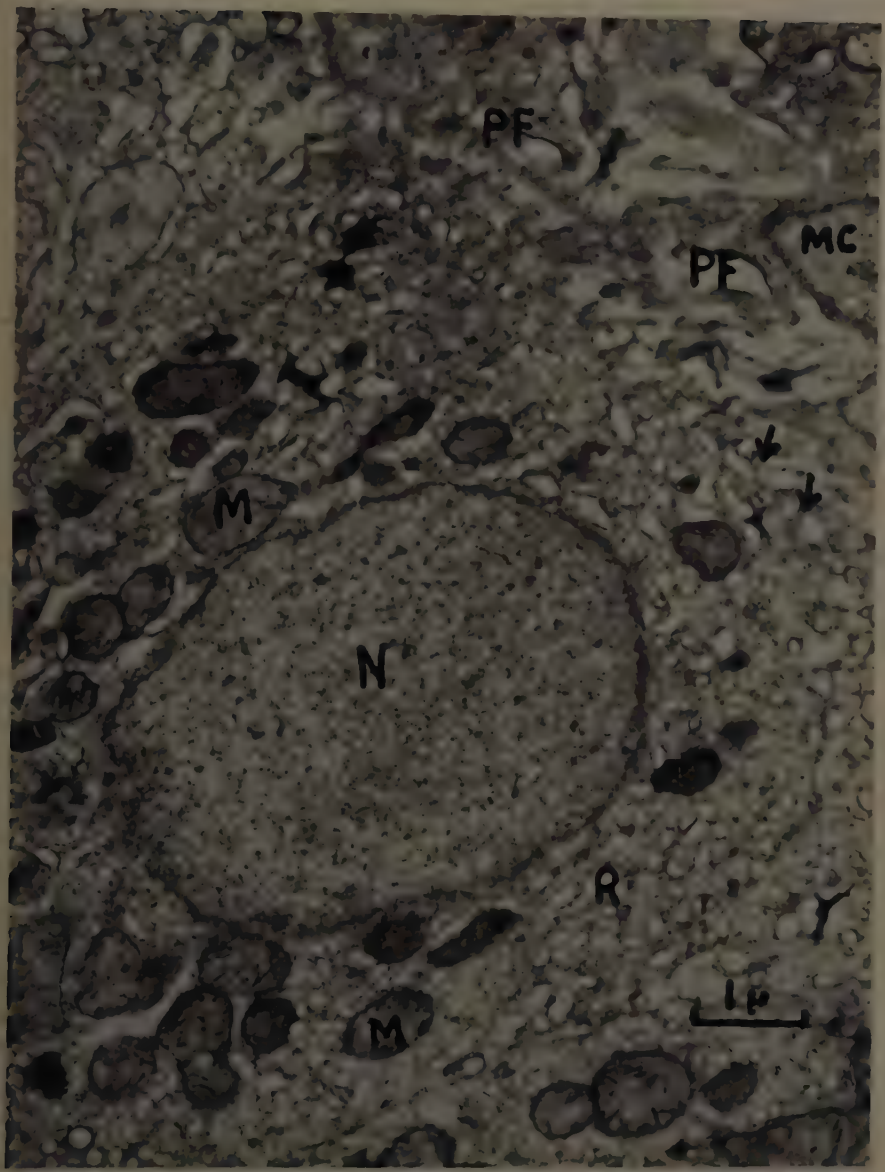

FIGURE 4. Electron photomicrograph of liver cell from a patient with chronic hepatitis, showing reduced numbers of mitochondria (M), small circular profiles of endoplasmic reticulum surrounding a nucleus (N) containing no nucleolus in the plane of section. The cell border with its microvilli (*arrows*) is covered by a thick layer of pericellular fibers (PF) in which is a mesenchymal cell whose nucleus (MC) is seen. R, reticulum. Osmium-fixed, methacrylate-embedded. ×16,000.

Kupffer's cell granules do not support such an assumption. Part of the paS-positive material is removed by prolonged lipid extraction. The Kupffer's cells give a strong acid phosphatase reaction. They have this characteristic in common with some peribiliary granules in the liver cells, which increase in size and amount in the presence of liver cell damage and probably represent the lysosomes, the organelles containing hydrolytic enzymes.[6] The increase of the paS-reaction of Kupffer's cell granules in any type of hepatic injury is independent of fibrosis and occurs in the presence of tissue breakdown anywhere in the body. It can be produced experimentally by injection of liver homogenates into the abdominal cavity. This indicates that these paS-positive granules are related to phagocytic activity of the Kupffer's cells, particularly since the paS and acid phosphatase reactions are histochemical features characteristic of reticuloendothelial cells in general. The sometimes very striking cytoplasmic basophilia of the Kupffer's cells and other reticuloendothelial cells of the liver is probably related to the formation of proteins other than collagen. This is supported by the observation that in some cases of human hepatic injury gamma globulin may be demonstrated by the Coons fluorescent antibody technique in cells that are apparently transitional between reticuloendothelial and plasma cells.[7] Liver cells injury thus is accompanied by fibrosis as well as by phagocytic and protein-forming activities of the surrounding mesenchyma. The ability of the hepatic mesenchyma to form ground substance and fibers still requires histochemical proof.

Periductular fibrosis. The normal bile ductule or cholangiole, the connection between liver cell plates and bile ducts in the portal tract, consists of cuboidal epithelial cells containing various amounts of fairly homogeneous cytoplasm and surrounded by a continuous silver-impregnated membrane even in thin sections. Under the electron microscope the ductular cells have fewer mitochondria and less ergastoplasm than do the parenchymal cells, but they have microvilli projecting into the lumen of the ductule (FIGURE 5). A continuous structureless basement membrane, not found around liver cells, separates them from the surrounding tissue. Outside of the membrane some mesenchymal cells and, occasionally, collagenous fibrils may be noted.

In many human liver diseases the ductules proliferate conspicuously and then frequently are surrounded by a thick layer of connective tissue (FIGURE 3*B*). Especially in postnecrotic cirrhosis, a great part of the fiber formation takes place around proliferating ductules. This is even more pronounced in hepatic fibrosis of rat produced in 7 weeks by a diet containing 0.5 per cent ethionine. In such rats and also in animals with some other types of experimental hepatic injury, excessive proliferation of interstitial cells is an important feature. These cells have been designated by various names. One is "oval cells,"[8] but retrograde injection of the biliary tree and observation of very thin sections clearly identify most of them as "ductular cells."[9] Also, on closer inspection, various mesenchymal cells, some obviously inflammatory and somewhat similar to those seen in the human liver, are noted around ductules. The bile ductules also in ethionine-intoxicated rats appear, with silver impregnation, to be surrounded by many fibers. Under the electron microscope the proliferated ductular cells in various forms of human cirrhosis, including hemo-

chromatosis, or in ethionine intoxication or other experimental injuries reveal the same microvilli projecting into the lumen as well as a similar basement membrane. On the outside of it, increased amounts of collagenous fibrils (FIGURE 6) are noted, which appear to be deposited on this basement mem-

FIGURE 5. Electron photomicrograph of normal rat liver ductule with 4 ductular cell nuclei (N) visible and a lumen (L) containing many microvilli. The arrows point to the thin basement membrane surrounding the ductule. Osmium-fixed, methacrylate-embedded. ×14,000.

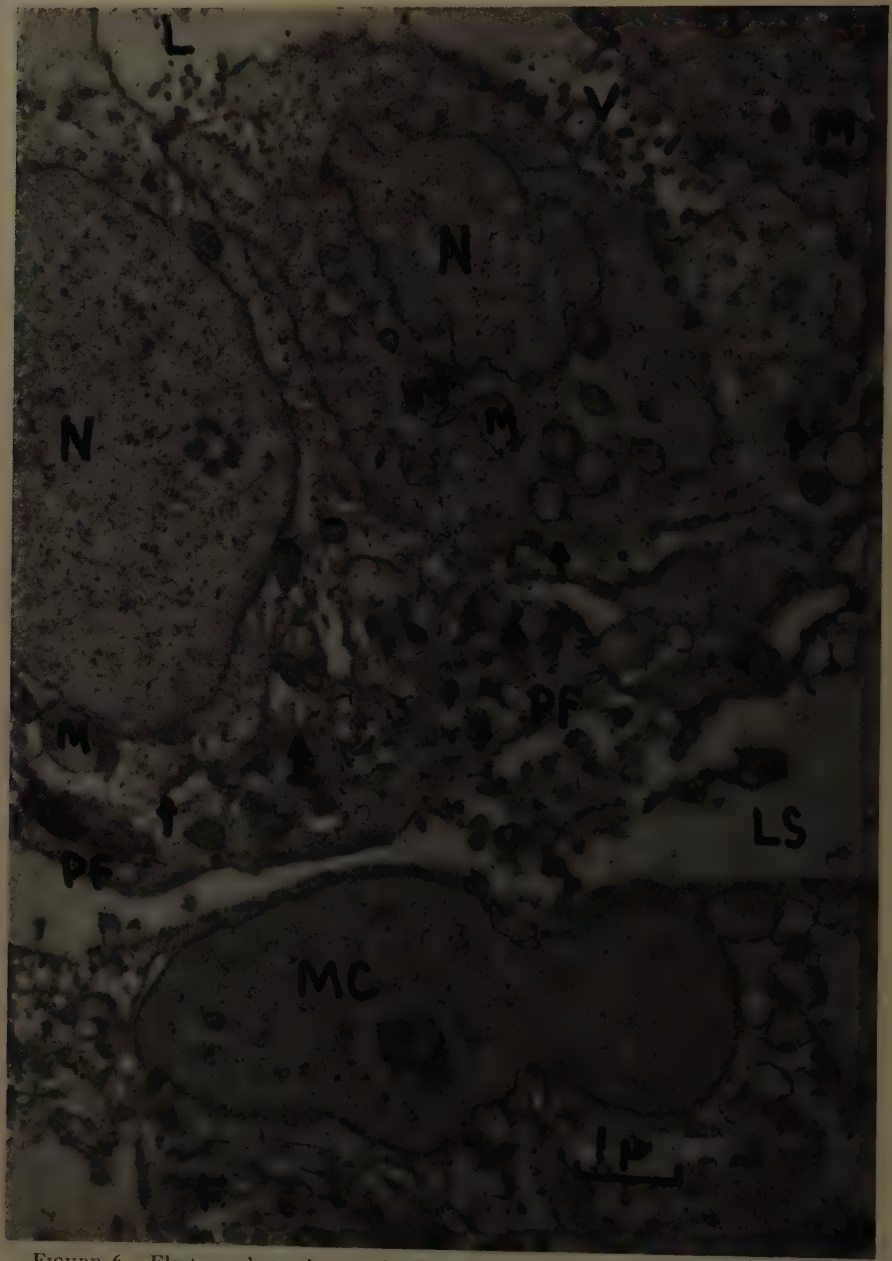

FIGURE 6. Electron photomicrograph of ductule from a rat with experimentally induced mild liver cell damage and ductular proliferation showing the ductular lumen (L) containing numerous microvilli (V) surrounded by ductular cells containing mitochondria (M) and nuclei (N) in the plane of the section in two. The basement membrane (*arrows*) is covered by an envelope of periductular fiber bundles (PF). A mesenchymal cell (MC) is in a lymph or tissue space (LS) and has many fibers (F) on the side away from the ductule. Osmium-fixed, methacrylate-embedded. ×14,000.

brane. A variable number and type of mesenchymal cells are seen nearby. In chronic processes such as human cirrhosis, fibroblasts may be conspicuous in the connective tissue septa separating the parenchyma. Although typical fibroblasts are not noted in acute stages of the experimental conditions, many of the surrounding mesenchymal cells contain paS-positive granules. In general, in both human and experimental material, the more active the fibrogenesis the more extensive the accumulation of mesenchymal cells around the ductules.

Portal fibrogenesis. The third type of fibrogenesis, which takes place in the portal tracts, shows the well-established features of fiber formation in connective tissue in general. Fibroblasts with or without paS-positive cytoplasm are noted in the more active conditions: for instance, in those associated with portal granulomas or prolonged bile stasis in which extracellular bile may act as stimulus. In more chronic types of portal fibrosis such as schistosomiasis, fibroblasts are in the background. An experimental illustration of portal fibroplasia is provided by the injection into the hepatic parenchyma of 0.5 ml. of a 0.3 per cent solution of carrageenin, a sulfated polygalactose from a seaweed (Irish moss). The resultant lesion resembles the well-studied carrageenin lesion in subcutaneous connective tissue.[10] Much paS-positive material, apparently ground substance, is laid down after an initial stage of necrosis. Fibroblasts with paS-positive cytoplasm and ductules proliferate rapidly, and the reaction is accompanied by other mesenchymal cells with paS-positive granules. This is associated with an excess of reticulum fibers, frequently in close approximation to the ductules.[11] Typically, the hepatic carrageenin granuloma, which is characterized by the presence of many mesenchymal cells disappears within 2 weeks, the rapid fibrogenesis being followed by a rapid fibrolysis. This reproducible course of the carrageenin granuloma is influenced by the same hormonal and vitamin factors as is the connective tissue formation after carrageenin injection into the subcutaneous tissue. For example, in scorbutic animals excessive ground substance accumulation is associated with delayed formation of fibers and local calcification.[12] Cortisone also has a specific altering effect.

Fibrosis in the portal tract under the electron microscope exhibits fibrils of the periodicity and width of other connective tissue. It deserves emphasis that, in the electron-microscope studies on hepatic fibrosis carried out thus far, the thickness and periodicity of the collagenous fibrils were the same throughout under the limits imposed by examination in embedded tissue. New formation of fibers therefore was characterized by excessive reduplication of preexisting fibers and development of thick bundles and sheets composed of elementary fibrils. Moreover, no difference was noted between fibers stained with collagen and those impregnated with silver, such as reticulum.

Chemical Aspects of Fibrogenesis

Attempts to study the chemical nature of the fibrogenesis in the liver are handicapped by the presence of large amounts of protein that are not scleroprotein in any fraction of the organ. If the scleroprotein cannot be separated from the large mass of other proteins, chemical changes in the former can be recognized only with difficulty. The demonstration of hydroxyproline not found in other hepatic proteins is the sole guide. In various types of cirrhosis,

both human and experimental, the total amount of hydroxyproline per liver is greatly increased, reflecting new formation of collagen.[13,14] When fat-free liver homogenates are treated with alkali (0.1 *N* NaOH), a soluble fraction of hepatic collagen can be separated. This fraction may include a precollagen[15] and may be the same as the fraction extractable in increased amounts from the connective tissue of animals exposed to the lathyrus factor (beta-aminopropionitrile).[16] This fraction was found to be increased in fetal liver and equally so in cirrhotic liver, in which its total amount rises to 10 times the normal, suggesting that some of the newly formed collagen is of this alkali-soluble variety[17] (TABLE 1). Ethionine-induced fibrosis is a useful model. In rats after 6 weeks on a 0.5 per cent ethionine diet the hepatic concentration as well as the total content of hydroxyproline rapidly increases to 3 to 5 times the normal, the increase being greater in the soluble fraction than in the insoluble one. This offers a reference for biochemical and histological correlation. The reticulum fiber increase is parallel with it. The same holds true for the ductular

TABLE 1
COLLAGEN DETERMINED FROM HYDROXYPROLINE CONTENT

	No. cases	Total (gm./100 gm. defatted liver)	Soluble (% of total)
Human			
Normal	13	2.90	10.5
Infants	15	1.15	24.1
Periportal fibrosis	7	3.30	
Subacute hepatitis	6	5.90	
Septal cirrhosis	17	10.20	22.6
Rat			
Normal	12	0.60	19.4
Subacute ethionine intoxication	9	1.95	31.4

cell proliferation, which is so excessive as to raise the liver weight to twice the initial value within a short time (FIGURE 7). All this is preceded by accumulation of paS-positive material in the third week, as well as by a parallel increase of total hepatic hexosamines and, somewhat later, by a considerable rise of proline in the soluble fraction without change of the total hepatic proline concentration.[18] This can be interpreted as accumulation of amino acid for transformation to hydroxyproline. During the same period changes in the activity of various enzymes occur, particularly a sharp drop in proline and, less conspicuously, in glutamic pyruvate transaminase oxidase.[19] However, further investigations are required to establish whether the fibrogenesis or the simultaneous alteration of the various hepatic cells is the important factor before any intriguing speculations as to the biochemical processes may be entertained.

Role of Cells in Fibrogenesis

Finally, the contribution of cells in the process of hepatic fibrogenesis deserves attention even if most of it remains in the realm of speculation. The

available information on fibrogenesis in general indicates[5,20] that fibroblasts probably produce the matrix and contribute at least the monomeric precursor of collagen, while the evidence that the collagenous fibril is fully developed within the cytoplasm of cells is not complete; it is assumed rather that this formation takes place on the cell surface.[20] Electron photomicrographs have been published showing collagenous fibrils with typical periodicity within mesenchymal cells of the normal[20,21] and the cirrhotic liver.[23] In our investigations fibrils have not been found within the cytoplasm of any cell, not even in rapid fibrogenesis induced by carrageenin. However, the liver probably is not the most favorable site for deciding whether collagenous fibrils are formed within or outside of cells, and the question of whether the published pictures

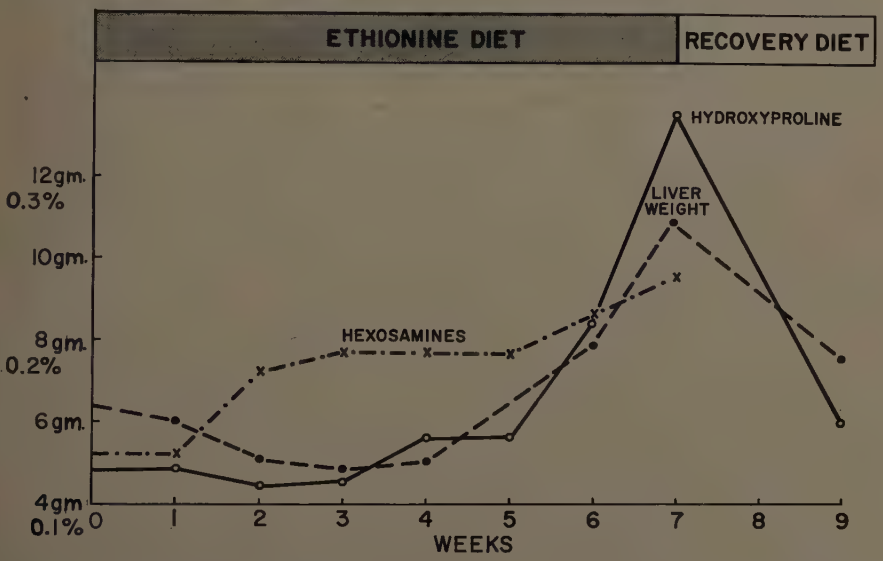

FIGURE 7. Concentration of hydroxyproline and total hexosamines and liver weight in rats on a synthetic diet containing 0.5 per cent ethionine, some rats after the seventh week receiving a synthetic diet containing 1.0 per cent methionine.

might represent artifacts is not discussed. In portal fibrogenesis, which is not the characteristic feature of the common liver diseases, fibroblasts seem to play much the same role as does any other part of connective tissue. On the other hand, in human and experimental perihepatocellular and periductular fibrogenesis typical fibroblasts are found only occasionally, whereas activation of mesenchymal cells of various types not necessarily fibroblastic in appearance is common.

For a better understanding of the relations, cell counts were made during active fibrogenesis, chiefly periductular fibrosis induced by ethionine. The counts were supplemented by determination of total hepatic content of hydroxyproline as well as of desoxyribonucleic acid (DNA), the latter as a measure of the total number of cells. In addition, when conspicuous fibrosis had developed after 7 weeks of a 0.5 per cent ethionine diet, rats were subjected either to a high-methio-

nine diet without ethionine or to cortisone administration. Both these procedures within 2 weeks resulted in rapid disappearance of the interstitial cell reaction consisting in proliferated ductular cells intermixed with mesenchymal cells and most of the excess reticulum fibers (FIGURE 8). During the active fibrogenesis, when the liver weight doubles, the total amount of liver cells hardly changes. However, mesenchymal cells including, for reasons of poor histological differentiation, Kupffer's cells, other reticuloendothelial cells, and inflammatory cells (portal tract tissue was not counted nor included in this consideration), increase to approximately 3 times the normal while ductular cells increase to about 80 times. During the same period hydroxyproline increases in parallel with DNA. After the recovery procedures, the liver weight

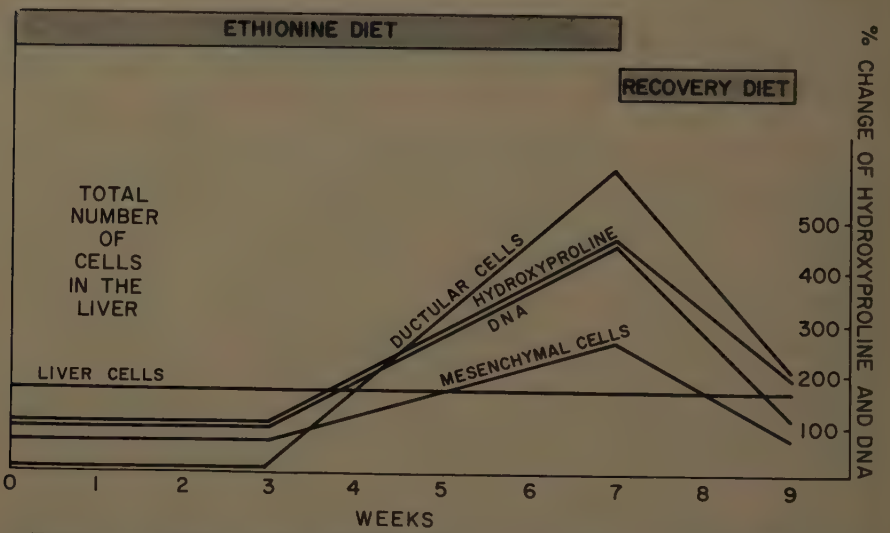

FIGURE 8. Relation of cell types expressed as total number of cells to change in total content of hydroxyproline and DNA in rats on a synthetic diet containing 0.5 per cent ethionine and subsequently on a synthetic diet containing 1.0 per cent methionine.

decreases to normal, the ductular cells are reduced to only 3 times the normal, DNA and mesenchymal cells return to normal, while the total hydroxyproline content remains somewhat higher than normal. These observations indicate that the increase in hydroxyproline as a reflection of fiber formation parallels an increase in the total number of cells. This might suggest that proliferation of either the ductular cells or the mesenchymal cells is related to the fiber formation.

Concluding Remarks

If at this time speculations are permitted that may serve as a working hypothesis, one might assume that the ductular cell proliferation reflects biliary excretion of a growth-promoting and, possibly also, irritating factor, particularly since in pilot experiments it is accompanied by a striking increase in the volume of a diluted bile. Possibly the excretion of the irritating material stimulates a

periductular inflammatory reaction typically seen in most human and experimental conditions with actively proliferating ductules. This in turn might stimulate fiber formation by the mesenchymal cells, the ductule acting as a nidus of collagen accumulation or, to use another term, a scaffold. However, in contrast to the carrageenin lesion in which abundant fibroblasts are seen during periductular fibrogenesis, ductules rather than the mesenchymal cells serve as a physical or directive force and might represent the limiting factor for the collagen formation. This means either increased activity of the few mesenchymal cells or reduced catabolism of the collagen fibrils in periductular fibrosis. With great caution, the supposition could be entertained that mesenchymal cells not only form collagen fibrils but also contribute to their disappearance, as suggested by the rapid dissolution of the carrageenin granuloma in the presence of many mesenchymal cells acting as "fibroclasts," to use an analogy from osteology. Whether the cells with the paS-positive granules, containing acid phosphatase and other hydrolytic enzymes, play this role is a question for further investigations. If so, possibly this fibrolytic activity is reduced in the presence of the ductular cells and thus becomes more efficient when the stimulus for ductular cell proliferation disappears as a result of recovery. Within two weeks the amount of fibers that are catabolized is four times that present in normal liver. Ductular cells as well as damaged liver cells thus might act as a scaffold for fiber formation.

Whatever the fate of these hypotheses, morphologic observation indicates that fiber formation in the abnormal liver takes place in three locations. In the commonest types of cirrhosis and chronic hepatitis the periductular and perihepatocellular fibrogenesis seem to be the more important, although the portal form may play some role, particularly in the production of portal hypertension. Both periductular and perihepatocellular fibroses are related to liver cell injury, which in turn seems to stimulate the mesenchymal reaction. For the present, the conclusion appears justified that avoidance and reversal of the parenchymal damage are the most promising methods of arresting and, possibly, reversing parenchymal fibrogenesis of the liver.

Acknowledgment

Appreciation is expressed to E. M. Rubin for the performance of the cell counts and histological gradings, to E. J. Singer for many biochemical analyses and F. G. Zak for supervision of the carrageenin experiments.

References

1. Popper, H. & H. Elias. 1955. Histogenesis of hepatic cirrhosis studied by the three-dimensional approach. Am. J. Pathol. **31:** 405.
2. Popper, H., F. Hutterer, G. Kent, H. M. van der Noen, F. Paronetto, F. Schaffner, E. J. Singer & F. G. Zak. 1958. Hepatic fibrosis: pathways and mechanism. J. Mt. Sinai Hosp. **25:** 378.
3. Popper, H., F. Paronetto, F. Schaffner & V. Perez. Studies on hepatic fibrosis. Lab. Invest. To be published.
4. Kent, G., I. G. Fels, A. Dubin & H. Popper. 1959. Collagen content based on hydroxyproline determinations in human and rat livers. Its relation to morphologically demonstrable reticulum and collagen fibers. Lab. Invest. **8:** 48.
5. Wassermann, F. 1959. The intercellular components of connective tissue, origin, structure and interrelationship of fibers and ground substance. Ergeb. Anat. u. Entwicklungsgeschichte. **35:** 240.

6. Novikoff, A. B. 1957. Biochemical heterogenicity of the cytoplasmic particles of rat. Symposium Soc. Exptl. Biol. **10:** 92.
7. Cohen, S., G. Ohta, E. J. Singer & H. Popper. Immunocytochemical study of gamma globulin liver in hepatitis and postnecrotic cirrhosis. J. Exptl. Med. In press.
8. Farber, E. 1956. Similarities in the sequence of early histological changes induced in the liver of the rat by ethionine, 2-acetylaminofluorene, and 3′-methyl-4-dimethylaminoazobenzene. Cancer Research. **16:** 142.
9. Popper, H., G. Kent & R. Stein. 1957. Ductular cell reaction in the liver in hepatic injury. J. Mt. Sinai Hosp. **24:** 551.
10. Williams, S. 1957. A histological study of the connective tissue reaction to carrageenin. J. Pathol. Bacteriol. **73:** 557.
11. Zak, F. G., I. Bubelis & F. Paronetto. 1958. Experimental fibroplasia in the liver induced by carrageenin in the normal rat and under the influence of cortisone and ethionine. Am. J. Pathol. **34:** 569.
12. Brazenas, N. J., F. Paronetto, A. W. Ludwig & F. G. Zak. 1958. Hepatic fibroplasia in normal and scorbutic guinea pigs. Am. J. Pathol. **35:** 696.
13. Morrione, T. G. 1947. Quantitative study of collagen content in experimental cirrhosis. J. Exptl. Med. **85:** 217.
14. Aterman, K. 1954. Studies in fibrosis of the liver induced by carbon tetrachloride. I. Relation between hepatocellular injury and new formation of fibrous tissue. A.M.A. Arch. Pathol. **57:** 1.
15. Orekhovitch, V. N. & V. O. Shpikiter. 1958. Procollagens. Science. **127:** 1371.
16. Levene, C. I. & J. Gross. 1959. Alterations in state of molecular aggregation of collagen induced in chick embryos by beta-aminopropionitrile (lathyrus factor). J. Exptl. Med. **110:** 771.
17. Hutterer, F., E. Rubin, E. J. Singer & H. Popper. 1959. Alkali-soluble and insoluble collagen in infant, adult and cirrhotic livers. Proc. Soc. Exptl. Biol. Med. **102:** 534.
18. Singer, E. J., F. Hutterer, G. Kent, F. G. Zak & H. Popper. 1959. Hepatic fibrosis. Chemical and histologic studies during subacute ethionine intoxication. A.M.A. Arch. Pathol. **68:** 103.
19. Hutterer, F. & E. J. Singer. 1959. Enzymatic aspects of hepatic fiber formation. Federation Proc. **18:** 482.
20. Porter, K. R. & G. D. Pappas. 1959. Collagen formation by fibroblasts of the chick embryo dermis. J. Biophys. and Biochem. Cytol. **5:** 153.
21. Wassermann, F. 1958. The structure of the wall of the hepatic sinusoids in the electron microscope. Z. Zellforsch. **49:** 13.
22. Cossel, L. 1959. Beitrag zur Ultrastruktur der Blutgewebsgrenze in der Leber. Elektronenmikroskopische Untersuchungen an Lebern von Mäusen und Leber Punktaten vom Menschen. Beitr. pathol. Anat. u. allgem. Pathol. **120:** 133.
23. Cachera, R. & F. Darnis. 1955. Examen du foie humain au microscope electronique. Semaine hôp. **31:** 2187.

CONNECTIVE TISSUE DISEASES AND CERTAIN SERUM PROTEIN COMPONENTS IN PATIENTS WITH AGAMMAGLOBULINEMIA*

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The clinical and laboratory evaluation of patients with agammaglobulinemia has provided immunological theory with information of fundamental significance. The most striking abnormality found is the inability of these patients to form circulating antibody.[1,2] Both electrophoretic and immunological tests show a virtual absence of gamma globulin.[1,2] Immunoelectrophoretic analysis has shown that two additional serum globulins are either absent or greatly diminished in these patients.[3-6] Both of these globulins are probably antibody globulins. This defect in antibody synthesis is expressed morphologically by the almost complete absence of plasma cells in the various hematopoietic tissues. After antigenic stimulation there is failure to form plasma cells or secondary nodules. The regular absence of secondary nodules is of interest in light of the recent demonstrations[7-10] of gamma globulin in the immature cells of the centers of such nodules. The defect in the hematopoietic tissues of the agammaglobulinemic patients appears to be of a somewhat primitive nature, however, since there is also among them a high incidence of such diseases as agranulocytosis[2] and alymphocytosis.[11] A number of instances of associative thymoma,[12] aregenerative anemia,[13] and granulomatous proliferation of the hematopoietic tissues[14] (R. A. Good and M. Karlin, unpublished observations) have been described. All these observations point to some defect of a more fundamental character than the simple inability to produce circulating antibody.

The basic genetic defect is expressed also in the high incidence of certain diseases of the connective tissues. Diseases such as tenosynovitis,[15] dermatomyositis[15] (also Hanson, personal communication), scleroderma,[16] rheumatoid arthritis, and diffuse fibrinoid vascular disease[17] have been observed with inordinate frequency in patients with agammaglobulinemia. A number of these different connective tissue diseases have been observed by Janeway *et al.*[15] in their agammaglobulinemic patients.

We have had the opportunity to study a total of 27 patients with agammaglobulinemia and, of these, 9 have had some form of connective tissue disease. With the exception of 1 patient, a child, this took the form of rheumatoid arthritis (TABLE 1). The child died of a diffuse vascular disease that terminated in acute renal failure. Early in the clinical course he was thought to have juvenile rheumatoid arthritis. He died prior to the identification of agammaglobulinemia in a younger sibling. A detailed review of the pathological material allowed the diagnosis of agammaglobulinemia. The fibrinoid lesions were thought to be most consistent with a diagnosis of diffuse fibrinoid vasculitis with thrombotic thrombocytopenic purpura (FIGURE 1).

* The investigation reported in this paper was supported in part by Research Grants and grants from the Minnesota Chapter of the Arthritis and Rheumatism Foundation, the Minnesota Heart Association and the American Heart Association, New York, N. Y.

All of the 8 other patients have had various classic manifestations of rheumatoid arthritis. They are grouped in TABLE 1 according to the diagnostic criteria of the American Rheumatism Association; the various manifestations are listed in TABLE 2. With one possible exception, morning stiffness was present in all of these patients. The possible exception (J.Sc.) had had a 4-week episode of arthritis at the age of 3. For this age such a history is of questionable validity. Multiple joints were involved in all of these patients, and, with one exception, symmetrical joint involvement was present. Probably of greater significance is the development of nodules in 3 of the 8 patients. In 2 patients these occurred over the extensor surface of the forearm. Two patients had multiple nodules, and one had multiple nodules involving the flexor tendons of the hand (FIGURE 2). At least 1 nodule was obtained at biopsy from each of these patients, and all nodules had the characteristic morphologic structure of the rheumatoid nodule (FIGURE 2). None of these patients has given positive test results for the rheumatoid factors when examined by a battery of techniques that has included agglutination reactions with

TABLE 1
AGAMMAGLOBULINEMIA AND CONNECTIVE TISSUE DISEASE

Total number of patients with agammaglobulinemia	27
Number with rheumatoid arthritis	
Classic	4
Definite	1
Probable	2
Possible	1
Number with other connective tissue disease	1
Total with connective tissue disease	9

both homologous and heterologous gamma globulin, FII precipitin tests, and euglobulin inhibition techniques. In the 2 patients who have had synovial biopsies, microscopic examination has revealed the proliferative and degenerative changes typical of rheumatoid arthritis. The exception in the characteristic histological changes has been the regular absence of plasma cells, although the other elements common to inflammatory reactions have been present. X-rays taken up to now have shown changes limited to demineralization about the involved joints in 3 of the 8 patients and to narrowing of the joint space in 2.

From the data presented, it seems clear that these patients suffer from a disease that is indistinguishable from rheumatoid arthritis. If this can be accepted as such, the implications are quite clear. The data of Holman and Kunkel (personal communication), showing the occurrence of frank rheumatoid arthritis, rheumatoid factor, both hypo- and hypergammaglobulinemia, and various other laboratory abnormalities in the families of patients with lupus erythematosus offer further evidence of a more fundamental defect than a simple derangement in antibody synthesis.

Recently we have encountered another facet of this disease that may be of importance to an understanding of the basic defect present in these patients. This has been the regular occurrence of certain $beta_2$ and gamma globulins

that are not demonstrable in normal individuals. These globulins have been discovered by the microimmunoelectrophoretic method of Scheidegger.[18] The method as used in our laboratory has been altered only in minor detail.[6] The nomenclature for the various bands follows that of Scheidegger,[18] and the des-

FIGURE 1. Section of kidney from patient with congenital agammaglobulinemia and fatal diffuse fibrinoid vasculitis. Hematoxylin and eosin.

TABLE 2
MANIFESTATIONS OF RHEUMATOID ARTHRITIS IN PATIENTS WITH AGAMMAGLOBULINEMIA

Manifestation	Congenital (age, color, sex)					Acquired (age, color, sex)		
	B.H., 10, WM	E.Si., 12, WM	D.R., 6, WM	J.Sc., 6, WM	T.G., 9, WM	L.L., 36, WF	F.H. 58, WM	E.K., 44, WF
(1) Morning stiffness	+	++	++	±	++	+++	++++	++
(2) Pain on motion or tenderness	++++	+++	+++	+++	++++	++	+++	+
(3) Swelling in one joint	++++	++++	++++	++++	++++	++++	++++	++
(4) Swelling in at least one other joint	+++	++++	+++	+++	+++	++++	++++	++
(5) Symmetrical joint swelling	−	++	+	++	++	++	+++	++
(6) Subcutaneous nodules	−	++	−	−	−	+	−	+
(7) X-ray changes typical of rheumatoid arthritis decalcification	−	+	−	−	−	+	±	−
(8) Agglutination	−	−	−	−	−	−	−	−
(9) Poor mucin precipitation from synovial fluid	not done	+	+	not done	not done	+	not done	not done
(10) Characteristic histological changes—joint	not done	++	+	not done	not done	not done	not done	not done
(11) Characteristic histological changes—nodules	not done	++	−	−	not done	++	not done	++++
(12) Duration (years)	2 Spont. remiss.	3½ Active	2 Active	4 wk. Spont. remiss.	6 wk. Spont. recur.	6 Remiss. with pregnancy, recur.	3½ Improv. with surg., remiss. with jaundice, dec'd.	4 Active

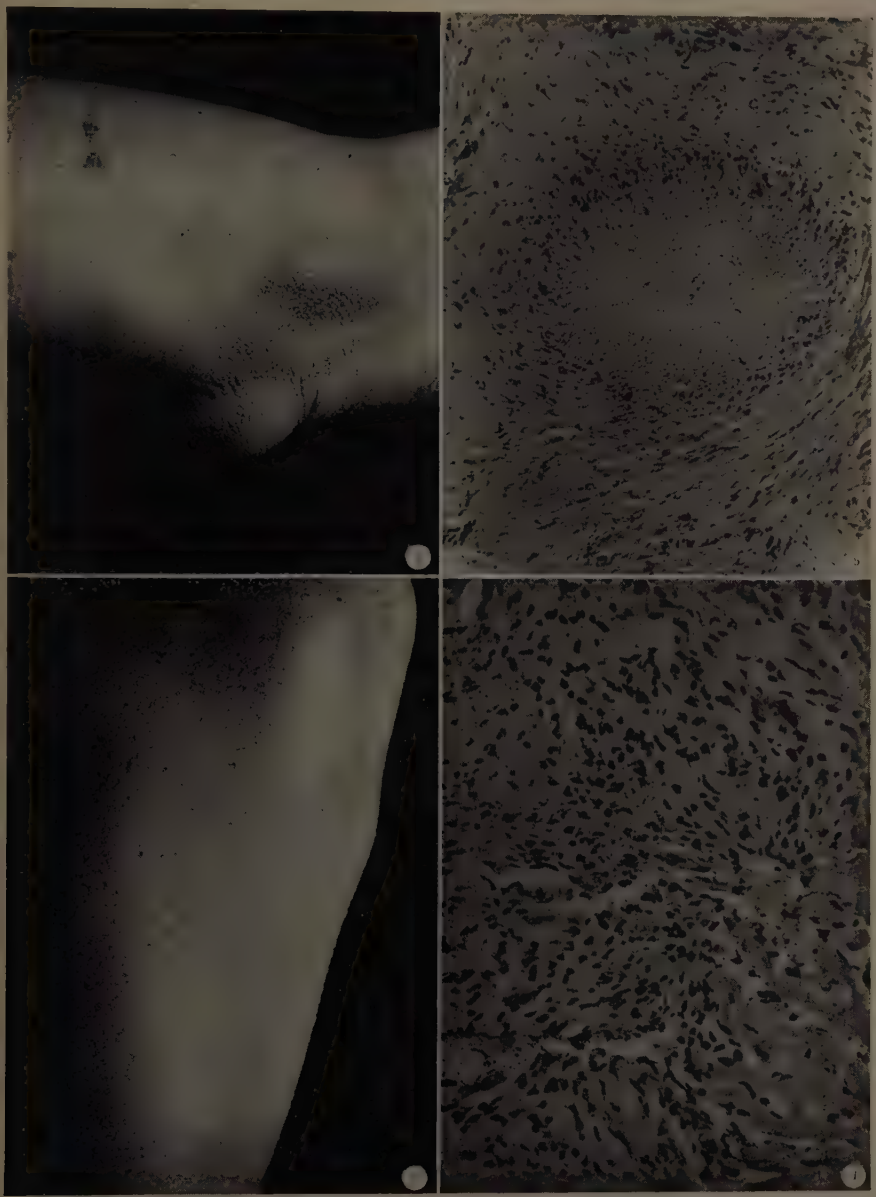

FIGURE 2. (*a*) Rheumatoid nodule at the elbow of a woman with acquired agammaglobulinemia. (*b*) Section from nodule shown in *a*. Hematoxylin and eosin. (*c*) Rheumatoid nodule on extensor surface of forearm in patient with congenital agammaglobulinemia. (*d*) Section from nodule shown in (*c*). Hematoxylin and eosin.

ignations for the bands reported here are intended to follow his scheme (FIGURE 3).

As shown in TABLE 3, three serum proteins not found in normal individuals are usually demonstrable in agammaglobulinemic patients. Of these the beta$_{2K}$ was found in all. This protein was seen in diseases other than agammaglobulinemia only in rare instances, although from a study of patients with

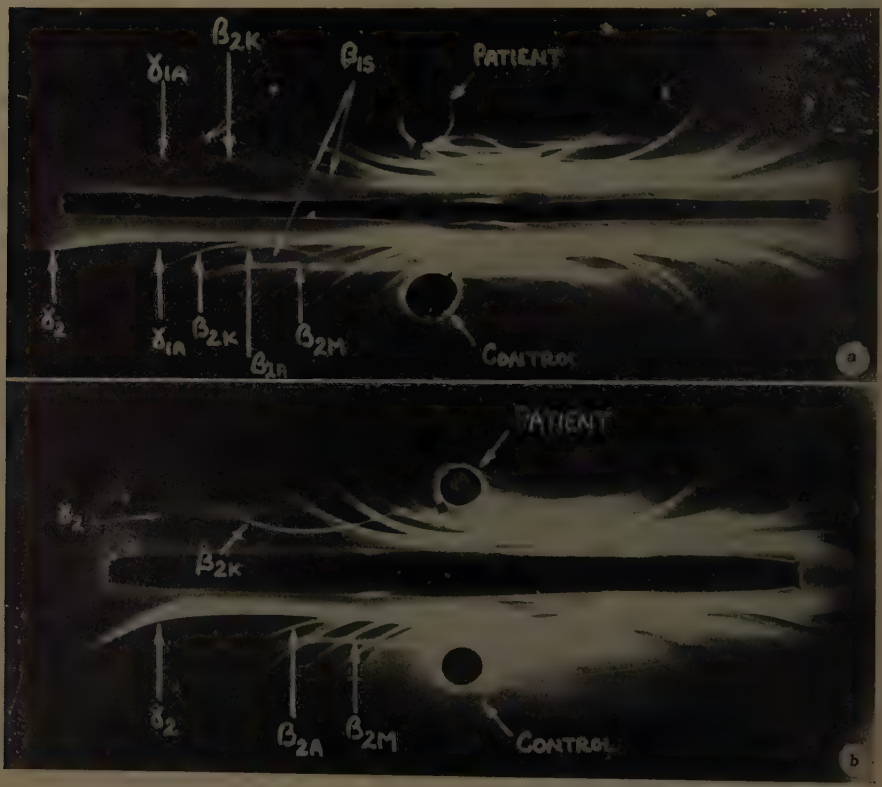

FIGURE 3. (*a*) Immunoelectrophoretic pattern showing gamma$_{1A}$ and beta$_{2K}$ in the serum from patient with congenital agammaglobulinemia. Further bands are identified in the control specimen on the opposite side. (*b*) Pattern of serum from patient with acquired agammaglobulinemia, showing heavy arc of beta$_{2K}$ precipitate. Control specimen on opposite side.

Hodgkin's disease (R. A. Bridges and R. A. Good, unpublished observations), it appears to have a similar high incidence. The beta$_{2E}$ (FIGURE 3) is another protein frequently evident in the serum from agammaglobulinemic patients that is only rarely found in patients with other diseases and never in healthy persons. This protein has been seen in 6 of 8 patients with congenital agammaglobulinemia and in 4 of 5 patients, with acquired agammaglobulinemia. Our studies commonly have revealed a third protein component that we have designated gamma$_{1A}$. This component has been encountered in approximately 50 per cent of patients with other diseases and thus appears to differ

in functional significance from the $beta_{2K}$ and the $beta_{2E}$. What is probably the same protein as $gamma_{1A}$ has been tentatively called $gamma_X$ by Heremans.[19]

In two patients further bizarre anomalies have been demonstrated by immunoelectrophoresis (FIGURE 3). Both patients have had multiple $beta_2$ and gamma globulin bands that appear distinctly different from any proteins pre-

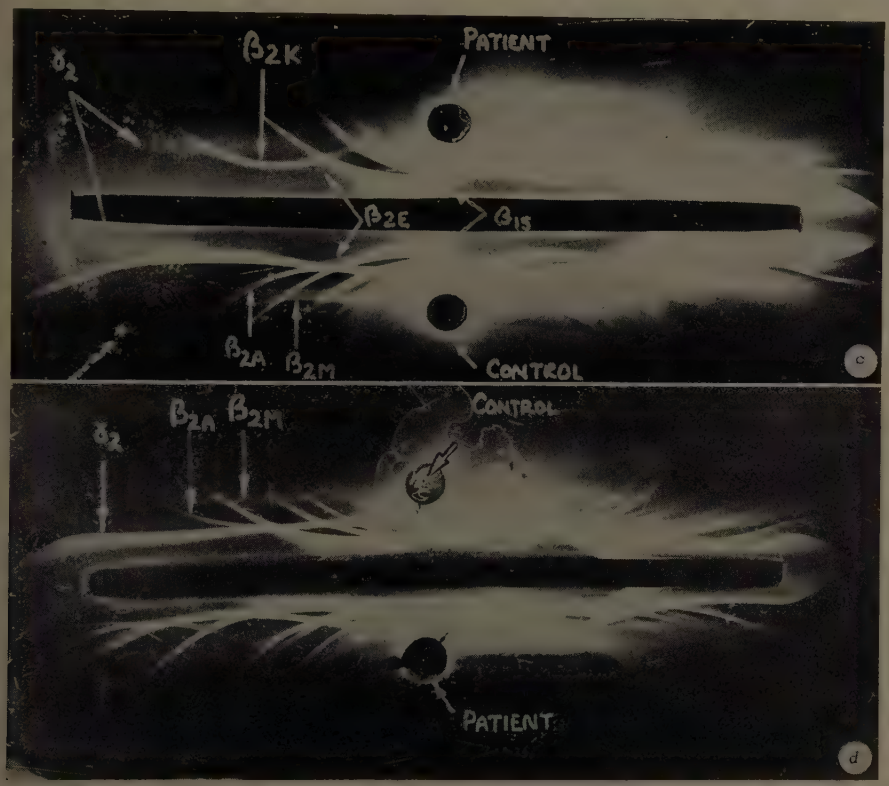

FIGURE 3. (*c*) Pattern of serum from patient with acquired agammaglobulinemia, showing $beta_{2E}$ and heavy arc of $beta_{2K}$. Control specimen on opposite side. (*d*) Pattern of serum from patient with congenital agammaglobulinemia, showing multiple anomalous $beta_2$ and gamma globulin bands. Control specimen on opposite side.

viously reported, except those of patients with multiple myeloma in whom such multiple bands are seen regularly[20] (R. A. Bridges and E. Freier, unpublished observations).

At least two of the protein components demonstrable in the $beta_2$ and $gamma_1$ globulin area of sera from patients with agammaglobulinemia may be related in some way to antibody synthesis. Since they have been demonstrated at this time by the use of antisera produced against either whole normal human serum or fractions of normal human serum, it seems probable that they are normal serum constituents that rise to demonstrable levels only under

very unusual circumstances, possibly appearing in the serum only when some derangement in antibody synthesis exists.

The various facets of the disease, agammaglobulinemia, emphasized here all point to a defect of fundamental importance. The extreme deficiency of gamma globulin so characteristic of these patients is only one of the numerous manifestations of the disease. A number of other diseases of the hematopoietic tissues have been reported as showing this deficiency of gamma globulin. It seems reasonable that with the elucidation of the pathogenetic mechanisms of these several diseases will come a fundamental explanation of the genetic defect in agammaglobulinemia.

TABLE 3

OCCURRENCE OF CERTAIN BETA$_2$ AND GAMMA$_1$ GLOBULINS IN PATIENTS WITH AGAMMAGLOBULINEMIA

Patient (year, color, sex)	Beta$_{2E}$	Beta$_{2K}$	Gamma$_{1A}$	Immunochemical gamma globulin (mg. %)
Congenital				
E.Si., 12, WM	+	+	+	3.0
J.Si., 3, WM	+	+	0	10
T.A., 4, WM	0	+	0	14
W.A., 10, WM	+	+	0	19
J.Sch., 3, WM	+	+	+	9
J.Sc., 6, WM	0	+	0	2.5
D.R., 6, WM	+	+	+	15
S.E., 13, WM	+	+	+	8
Acquired				
L.L., 36, WF	+	+	+	6
E.K., 44, WF	0	+	+	42
B.A., 75, WF	+	+	0	74
F.H., 58, WM	+	+	+	81
L.Z., 57, WF	0	+	+	33

References

1. JANEWAY, C. A., L. APT & D. GITLIN. 1953. Agammaglobulinemia. Trans. Assoc. Am. Physicians. **66:** 200.
2. GOOD, R. A. & S. J. ZAK. 1956. Disturbances in gamma globulin synthesis as "experiments of nature." Pediatrics. **18:** 109.
3. GITLIN, D., W. H. HITZIG & C. A. JANEWAY. 1956. Multiple serum protein deficiencies in congenital and acquired agammaglobulinemia. J. Clin. Invest. **35:** 1199.
4. SCHEIDEGGER, J. J. 1956. L'immuno-électrophorèse. Semaine hôp. **32:** 2119.
5. GRABAR, P., P. BURTIN & M. SELIGMANN. 1958. Études immuno-électrophorétiques et immunochimiques des agammaglobulinémies. Rév. franç. études clin. biol. **3:** 41.
6. BRIDGES, R. A. & R. A. GOOD. The immunoelectrophoretic characterization of agammaglobulinemic states. J. Clin. Invest. In press.
7. WHITE, R. G. 1959. The relation of the cellular responses in the "germinal or lymphocytopoietic" centres of lymphoid tissue to the production of antibody. *In* Symposium on Mechanism of Antibody Formation. : 2. Abstracts. Prague.
8. ORTEGA, L. G. & R. C. MELLORS. 1957. Cellular sites of formation of gamma globulin. J. Exptl. Med. **106:** 627.
9. MELLORS, R. C., R. HEIMER, J. CORCOS & L. KORNGOLD. 1959. Cellular origin of rheumatoid factor. J. Exptl. Med. **110:** 875.
10. KAPLAN, M. H., M. L. SUCHY & M. MEYESERIAN. 1959. Composition of precipitates formed between rheumatoid sera and dye-labelled F-II reactant (abstract). Proc. 6th Interim Session Am. Rheumatic Assoc. Detroit, Mich.

11. Young, I. I., W. Q. Wolfson & C. Cohn. 1955. Studies in serum proteins; agammaglobulinemia in the adult. Am. J. Med. **19:** 222.
12. MacLean, L. D., S. J. Zak, R. L. Varco & R. A. Good. 1957. The role of the thymus in antibody production: an experimental study of the immune response in thymectomized rabbits. Transplantation Bull. **4:** 21.
13. Ramos, A. J. 1956. Presentation of a case, with discussion by V. Loeb. J. Am. Med. Assoc. **160:** 1317.
14. Zinneman, H. H. & W. H. Hall. 1956. Steatorrhea and probable tuberculosis with acquired hypogammaglobulinemia. Am. Rev. Tuberc. **74:** 773.
15. Janeway, C. A., D. Gitlin, J. M. Craig & D. S. Grice. 1956. Collagen disease in patients with congenital agammaglobulinemia. Trans. Assoc. Am. Physicians. **69:** 93.
16. Van Gelder, D. W. 1957. Clinical significance of alterations in gamma globulin levels. Southern Med. J. **50:** 43.
17. Good, R. A., J. Rotstein & W. F. Mazzitello. 1957. The simultaneous occurrence of rheumatoid arthritis and agammaglobulinemia. J. Lab. Clin. Med. **49:** 343.
18. Scheidegger, J. J. 1955. Une micro-méthode de l'immuno-électrophorèse. Intern. Arch. Allergy. **7:** 103.
19. Heremans, J. F. 1959. Immunochemical studies on protein pathology. The immunoglobulin concept. Clin. chim. Acta. **4:** 634.
20. Grabar, P., R. Fauvert, P. Burtin & L. Hartmann. 1956. Étude sur les protéines du myélome. II. L'analysis immuno-électrophoretique des serums de 30 malades. Rév. franç. études clin. biol. **1:** 175.

Discussion

Gustave J. Dammin (*Peter Bent Brigham Hospital, Boston, Mass.*): Some years ago your group reported prolonged persistence of skin grafts in patients with congenital agammaglobulinemia, irrespective of blood group and sex differences between donor and recipient. What has been the later course of these skin grafts?

Bridges: The skin grafts are still intact.

HEREDITY AND DISEASES OF CONNECTIVE TISSUE*

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As in the case of other systems, analysis of genetic factors in diseases of the connective tissue system tends to divide these disorders logically into two parts: (1) less common disorders due primarily to a single mutant gene, for example, the Marfan syndrome; and (2) the relatively common disorders in which genetic factors may be significantly contributory in the etiology or pathogenesis, for example, rheumatoid arthritis.

Five Generalized and Heritable Disorers of Connective Tissue

Marfan syndrome. This syndrome (FIGURE 1) is manifested by changes in the eye, especially ectopia lentis (subluxation of the lens); in the skeleton, especially excessive length of the round bones of the extremities; and in the cardiovascular system, especially a weakness of the aortic media leading to diffuse aneurysm, dissecting aneurysm, or a combination of the two. Loose-jointedness and hernia are often conspicuous.

The pedigree data are consistent with transmission of the Marfan trait as an autosomal dominant.

Cases with only skeletal features to indicate the Marfan syndrome cannot be diagnosed with certainty if there is not even the slightest abnormality of the suspensory ligament of the lens and if there are no more definite cases in the family. In an attempt to quantify the dolichostenomelia we have obtained normal standards for upper segment–lower segment ratio (US:LS), the segments being measured above and below the top of the pubic symphysis (FIGURE 2A to C). The old data of Engelbach,[1] which is very extensive but was collected between 30 and 70 years ago, is not now applicable. Interestingly, Negroes examined, both male and female, have lower US:LS ratios at all ages. When cases of the Marfan syndrome are compared with these normal standards, it is found that their ratios fall on the low side of normal. However, because of the overlap with the normal, the US:LS is scarcely a pathognomonic test.

What is the nature of the gene-determined defect in connective tissue in the Marfan syndrome? The dramatic gross and histological changes in the ascending aorta, behaving like a wearing-out process, might suggest that the defect is intimately concerned with the elastic fiber. How does a connective tissue defect lead to excessive longitudinal growth of bones? There is the impression that longitudinal growth is unreined. One is reminded of the old experiments of Ollier indicating that normally the periosteum, by its connections to the epiphyses, is dragged along the surface of the elongating diaphysis and seems to exercise some control over longitudinal growth. Although a defect of the connective tissue of periosteum may be involved in the dolicho-

* A more comprehensive discussion of these problems, together with a review of the literature, is presented in the following two publications: McKusick, V. A. 1960. *Heritable Disorders of Connective Tissue.* 2nd ed. Mosby. St. Louis, Mo.; McKusick, V. A. 1959. *Genetic factors in diseases of connective tissue.* Am. J. Med. **26:** 283.

FIGURE 1. The Marfan syndrome. The older brother (*right*) has no ocular or cardiovascular anomaly although the skeletal features, especially pectus excavatum, are pronounced. In him the diagnosis of the Marfan syndrome could not have been made with confidence but for the facts that his younger brother (*left*) does have ectopia lentis and even more marked skeletal features and that his father died at 36 years of aortic regurgitation.

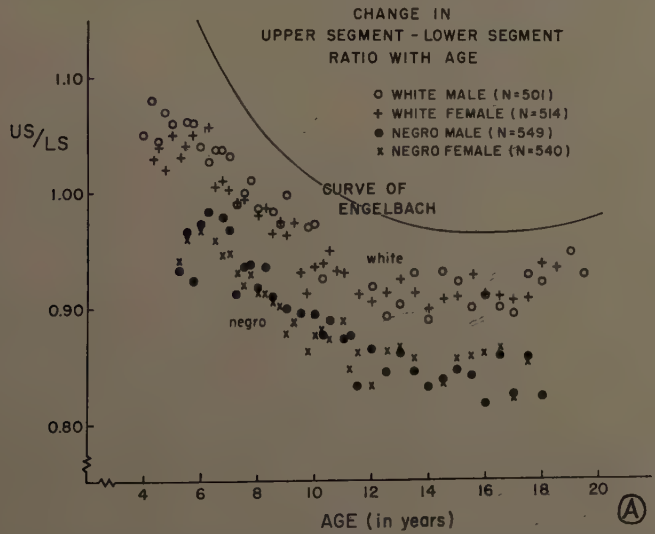

FIGURE 2*A*. Upper segment:lower segment ratio. Data collected in "normal" Baltimore school children demonstrate (1) that the old data of Engelbach are no longer applicable, and (2) that Negroes of both sexes and all ages studied have a lower mean US:LS than do whites. Analysis reveals that the lower mean US:LS of the Negro is due in part to a shorter upper segment and in part to a longer lower segment.

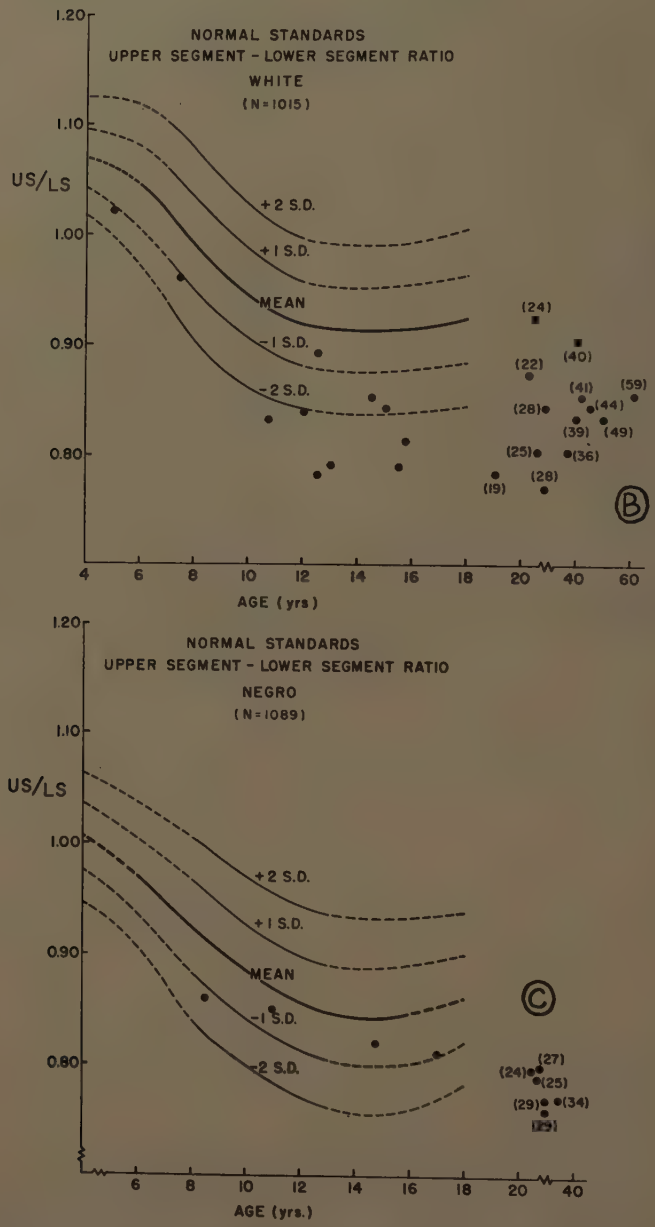

FIGURE 2*B* and *C*. Upper segment:lower segment ratio. In patients with the Marfan syndrome (*dots*) the US:LS tends to be low, but the overlap with the normal is considerable (the age of each older subject is given in brackets).

stenomelia, the unfortunate situation is that there is no precise information about the defect in the ligaments, fascia, tendons, aortic media, and lens ligament, resulting in the several features of the Marfan syndrome.

James T. Leeming and I have followed up the suggestion of Bacchus that the Winzler serum mucoproteins are abnormally low in the Marfan syndrome. In 40 indubitable cases of the Marfan syndrome distributed through a total of 24 families, as well as in 14 doubtful cases, no significant difference could be demonstrated from non-Marfan controls matched for age, sex, and race (TABLE 1).

Ehlers-Danlos syndrome. In full-blown form this syndrome (E-D) is manifested by changes in the joints, particularly hypermobility; in the skin, by unusual stretchability (ordinarily without loss of elasticity), fragility, bruisability, and peculiar scarring; internally, by diverticula of the gastrointestinal tract, hiatus hernia, and eventration of the diaphragm. Involvement of the heart and aorta is becoming recognized. In its milder form the disease is

TABLE 1

SERUM MUCOPROTEINS IN THE MARFAN SYNDROME AND IN MATCHED CONTROLS
Determined by Modification of Turbidimetric Method of de la Huerga *et al.*[2]

	White				Colored			
	Male		Female		Male		Female	
	Control	Marfan	Control	Marfan	Control	Marfan	Control	Marfan
Number of persons	28	17	14	9	29	9	17	5
Mean mucoprotein level (mg. %)	155.67	167.02	146.01	179.10	142.56	170.66	132.95	141.84
S. D.	29.32	24.70	26.78	59.22	29.24	44.77	19.54	32.40
S. E. (mean)	5.54	5.99	7.16	19.74	5.43	14.92	4.74	14.49

difficult to diagnose with assurance. Hypermobility of joints and stretchability of skin are graded characteristics.

It is unclear what relation to E-D is borne by the occasional case of simple joint hypermobility without fragility of the skin, easy bruisability, and "cigarette-paper" scars, probably without internal involvement (FIGURE 3).

Pedigree information is consistent with transmission of E-D as an autosomal dominant.

The theory of Jansen,[3] that the defect in E-D involves the weaving of collagen bundles into a normal wickerwork, requires further corroboration. Soon after pancreatic elastase was discovered, an inhibitor in normal serum was also described. The report of an increase in serum elastase inhibitor in two cases of E-D is worthy of further study.

Osteogenesis imperfecta. This disorder (OI) is not simply a disease of bone, although brittle and soft bones usually are the most impressive and important features. Not only is the organic matrix of bone defective but also the collagen of sclera, leading to thin "blue sclerotics"; of skin that is thin and produces broad surgical scars; of fascia leading to hernia; and of ligaments, tendons,

FIGURE 3A. Simple joint hyperextensibility. This 28-year-old woman displays hyperextensibility of the knees and elbows and loose-jointedness at other sites, but no unusual stretchability, bruisability, fragility, or scarring of the skin. The mother, at least one sibling, and one offspring are similarly affected.

FIGURE 3B. Simple joint hyperextensibility. See legend FIGURE 3A.

and joint capsules leading to loose-jointedness. The deafness, usually referred to as "otosclerosis," appears to result from the same connective tissue defect in the bones and soft tissues in and around the middle and inner ear.

The defect in OI rather clearly involves collagen. It is to be hoped that studies of the physical and chemical nature of collagen in this and other heritable disorders of connective tissue will be undertaken by those competent to do so: it is possible that the collagen in OI is anomalous in its amino acid sequence with an amino acid substitution as specific as those pinpointed[4] in several of the variant hemoglobins.

Classically, OI is transmitted as an autosomal dominant. It seems likely that the cases of profound involvement already present at birth in the offspring of normal parents, so-called congenital OI, are new dominant mutations at the same genetic locus, but the existence of a recessive form of the disease is possible. In fact, there is no proof that several distinct entities are not involved in the condition we label OI.

Pseudoxanthoma elasticum. By nature this disorder (PXE) is an abiotrophy. Although the fundamental defect of connective tissue is undoubtedly present from the beginning, the deterioration that makes its presence clinically evident does not occur until a few years or even a few decades after birth. The skin of the flexoral areas—neck, axilla, groin, and the like—becomes lax, grooved, ridged, and slightly nodular. Crazing of Bruch's membrane behind the retina, expresses itself as angioid streaking detected by funduscopy. The media of arteries of intermediate and smaller size also undergo degeneration, showing premature calcification, peripheral arterial insufficiency, angina pectoris, hypertension and, especially, a proneness to gastrointestinal hemorrhage.

Pedigree evidence is consistent with the transmission of PXE as an autosomal recessive.

Histological studies of skin, endocardium, and small blood vessels show fragmentation of connective tissue fibers with resulting granular and irregularly shaped bits that tend to stain with "elastic stains" and have a pronounced affinity for the calcium ion. Whether the degenerate connective tissue fibers are derived from collagen or from elastic fibers is unsettled, in my opinion, there being evidence for each view.

Hurler syndrome. It has been demonstrated in recent years that in this syndrome (gargoylism) large amounts of two mucopolysaccharides, chondroitin sulfate B and heparitin sulfate, are excreted in the urine and accumulate in certain tissues such as the liver, spleen, meninges, and vascular intima. Characteristic skeletal deformity, stiff joints, hepatosplenomegaly, cloudy cornea, angina pectoris, and valvular heart disease are the clinical manifestations. Impairment of intellect is partially due to hydrocephalus from involvement of the meninges, but other mechanisms not now understood must be involved.

There are two genetic forms of the disease. The more frequent, inherited as an autosomal recessive, is also clinically more severe. In the rarer sex-linked recessive form, clouding of the cornea probably never occurs and the victim may survive to his forties. Interestingly, the pattern of mucopolysaccharide excretion in the urine is not different in the two genetic varieties.

For this reason and since two mucopolysaccharides are involved, the basic defect must be located several steps proximal to chondroitin sulfate B and heparitin sulfate, at least proximal to a fork in the metabolic road leading to these two mucopolysaccharides. A. Dorfman (personal communication) recently made the interesting suggestion that the protein binding of these mucopolysaccharides may be defective.

A number of other conditions, single gene defects of connective tissue, will not be cited for lack of space. Obviously, much remains to be learned about the nature of the basic defect in each of the heritable disorders of connective tissue. There is also still room for further clinical analysis, especially in sorting out the confusing chondrodystrophies: those to which the Morquio eponym is attached and others. One cannot but think that much of basic interest will be learned about connective tissue in the process of investigating the basic defects.

The Common Diseases of the Connective Tissue System

A familial aggregation that may have a genetic basis has been demonstrated in rheumatoid arthritis, in ankylosing spondylitis, and in rheumatic fever. Indeed, a simple Mendelian hypothesis to explain transmission of susceptibility has been advanced in the case of each. Most genetic studies of systemic lupus erythematosus reported thus far have concerned isolated families. Whether there is a significant familial aggregation in this disease must be considered still an open question.

In diseases such as these, in which environmental factors and genetic factors probably collaborate, genetic analysis is difficult. I can see no hope of significant progress in this analysis until more is known about the basic mechanisms. As each new biochemical, serologic, or other peculiarity of these diseases is uncovered, its distribution in families and in monozygotic versus dizygotic twins should be determined. Lawrence and Ball[5] in Manchester and Ziff and his colleagues[6] in the United States, for example, have made revealing family studies of rheumatoid arthritis based on tests for the rheumatoid factor.

Family studies can reveal a pathogenetic relationship between two diseases when an impressive frequency of one disease is found in families studied because of a proband with a second disease. One example of this use of family studies is the demonstration of an increased frequency of spina bifida among the sibs of anencephalic stillbirths, suggesting a pathogenetic relationship between the two malformations. Kunkel and his colleagues may be demonstrating a fundamental relationship between rheumatoid arthritis and systemic lupus when, as they recently reported in a preliminary paper,[7] they find among relatives of systemic lupus probands an impressive incidence of clinical rheumatoid arthritis and of seropositivity in tests for rheumatoid factor.

The genetics of osteoarthritis also are not investigated easily. Occasionally a familial predisposition to precocious osteoarthritis can be shown to have its basis in hereditary epiphyseal dysplasia or in other clearly genetic disorders (FIGURE 4). This, however, is secondary osteoarthritis and represents a minority of cases.

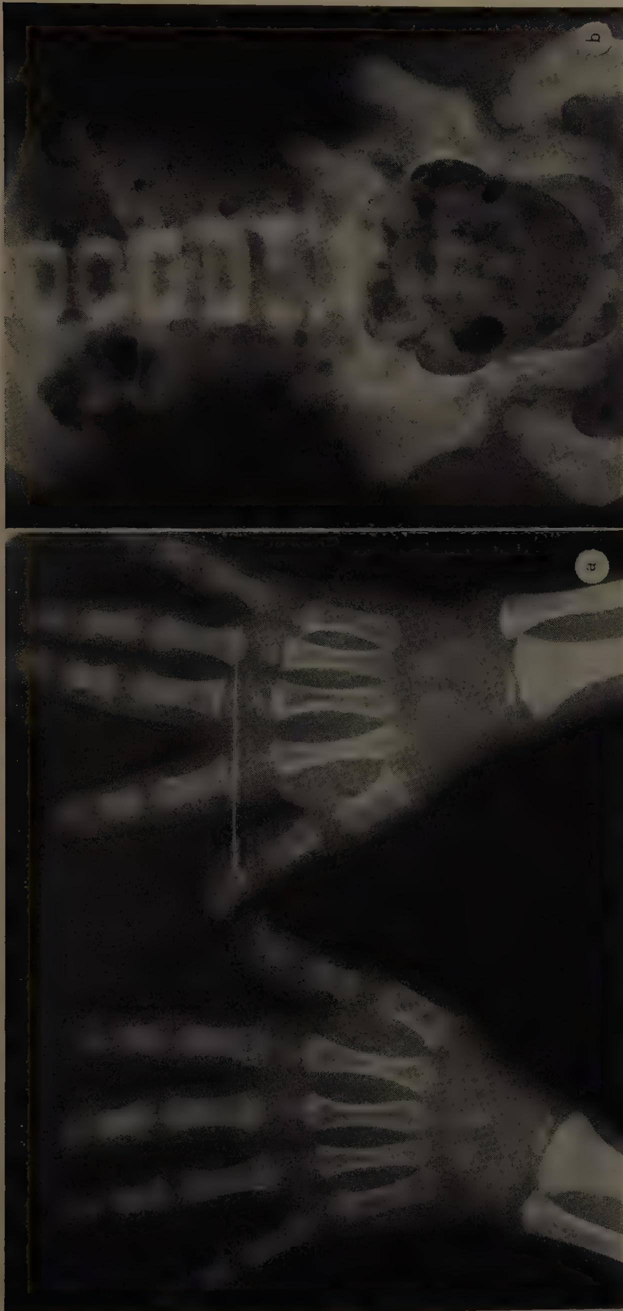

FIGURE 4A and B. Multiple epiphyseal dysplasia in father and son with secondary osteoarthritis in the father. Hands and pelvis in H.W.C., Jr., aged 9 years. A brother of the father and the grandfather are similarly affected. Furthermore, there are several other affected members of the family, cousins of H.W.C., Jr. In so far as the pedigree has been investigated, the pattern of inheritance is consistent with autosomal dominance. Affected adults are of short stature.

FIGURE 4*C* and *D*. Multiple epiphyseal dysplasia in father and son with secondary osteoarthritis in the father. Hands and pelvis in H.W.C., Sr., aged 40 years. See FIGURE 4*A* and *B*.

Two "Honorary" Diseases of Connective Tissue

There are at least two inborn errors of metabolism that are, one might say, honorary diseases of connective tissue: alkaptonuria and gout.

One of the more interesting recent demonstrations concerning the genetics of alkaptonuria is that of my colleague Milch,[8] who showed that the seemingly dominant form of the disease is probably the usual recessive form appearing in successive generations through the mating of homozygous affected persons with heterozygous carriers. Consanguineous matings increase the likelihood of this occurrence.

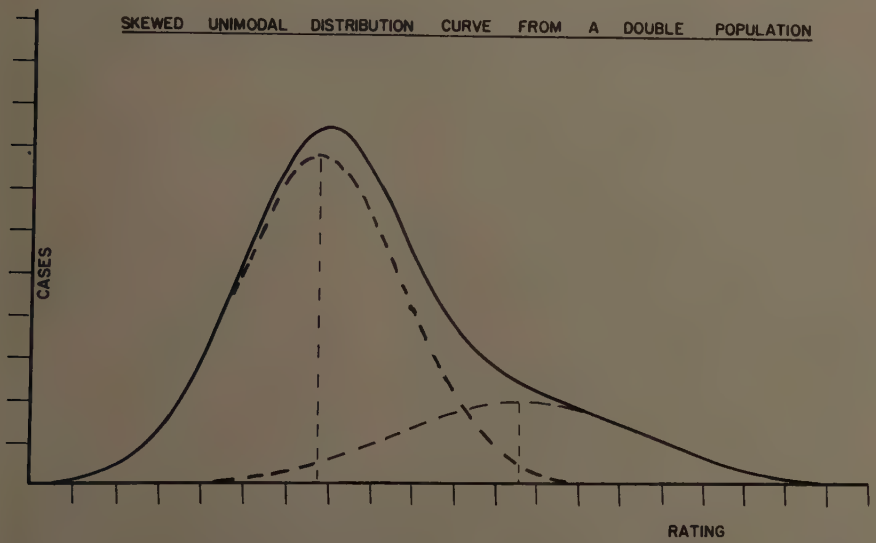

FIGURE 5. Skewed unimodal distribution curve from a double population. As Gaddum has pointed out, many biological variables (for example, systolic or diastolic blood pressure fasting blood glucose, and serum uric acid) display a positively skewed unimodal frequency distribution. The question is: Are there, in fact, two populations making up said distribution? Reproduced by courtesy of E. A. Murphy.

In gout the most interesting genetic question is whether a single mutant gene is responsible or whether hyperuricemia (and therefore gout) is a polygenic character. The frequency distribution of serum uric acid is "continuous," that is, unimodal. The suspicion is that a serum uric acid level is the net result not only of environmental factors, notably the amount of protein consumed, but also of multiple genetic factors; that serum uric acid in its genetics falls into the same pattern as stature, intelligence, ocular refraction, and many other characteristics. The overproduction theory of primary hyperuricemia seems well established. The polygenic basis of hyperuricemia is attractive because there is more than one step in the synthesis of uric acid, each presumably under separate enzymatic (and genetic) control. However, plausibility is not proof. There may still be a single major gene responsible for hyperuricemia, and the expected bimodality may be blurred (FIGURE 5) by the dietary factors and by the age effect. The secondary mode would not be

expected to be large, considering the low frequency of gout, and would be concealed rather easily. In fact, Smyth *et al.*[10] appear to have demonstrated bimodality for level of serum uric acid in the first-degree relatives of gouty probands. Such relatives are an informative group for examination of the question of bimodality since, according to the theory of autosomal dominance, one half may be expected to have the gene determining hyperuricemia.

The question of polygenic inheritance versus monogenic inheritance is of more than academic interest. The whole outlook on diseases such as hypertension, hypercholesteremia, and hyperuricemia is different when there is evidence of a major unifactorial genetic basis: one may infer a unitary biochemical defect that may be sought and, if found, may be the basis of preclinical diagnosis, definitive therapy, and prophylaxis.

Chlorothiazide and its congeners cause a rise in serum uric acid through a renal mechanism. I have great hopes that the use of chlorothiazide in family studies of gout will improve the phenotypic classifications. Presumably, when a dam is thrown up at the renal level, serum uric acid will rise higher in those persons with the genotype for overproduction of uric acid. On the hypothesis of a single dominant gene, the first-degree relatives of a gouty proband—sibs, for example—may be expected to fall into two groups, those with and those without the gene, those with the gene showing an exaggerated rise of serum uric acid when chlorothiazide is given. More data are needed before we can say whether the facts bear out the theory. Other possible methods for improved phenotype classification are: (1) the administration of a uric acid precursor such as ribonucleic acid, or (2) the measurement of some other blood constituent as, for example, oxalate.[11]

References

1. Engelbach, E. 1932. Endocrine Medicine. Thomas. Springfield, Ill.
2. de la Huerga, J., A. Dubin, D. S. Kushner, H. A. Dyniewicz & H. Popper. 1956. Studies of serum mucoprotein (seromucoid). I. A turbidimetric method. J. Lab. Clin. Med. **47:** 403.
3. Jansen, L. H. 1955. The structure of the connective tissue, an explanation of the symptoms of the Ehlers-Danlos syndrome. Dermatologia. **110:** 108.
4. Ingram, F. M. 1959. Chemistry of the abnormal human hemoglobins. Brit. Med. Bull. **15:** 27.
5. Lawrence, J. S. & J. Ball. 1958. Genetic studies in rheumatoid arthritis. Ann. Rheumatic Diseases. **17:** 160.
6. Ziff, M., R. F. Schmid, A. J. Lewis & M. Tanner. 1958. Familial occurrence of the rheumatoid factor. Arthritis and Rheumatism. **1:** 392.
7. Kunkel, H. G. 1959. Immunologic aspects of rheumatoid arthritis. J. Chronic Disease. **10:** 418.
8. Milch, R. A. 1959. A preliminary note on 47 cases of alkaptonuria occurring in seven interrelated Dominican families, with an additional comment on two previously reported pedigrees. Acta Genet. et Stat. Med. **9:** 123.
9. Gaddum, J. H. 1945. Lognormal distributions. Nature. **156:** 463.
10. Smyth, C. J., C. W. Cotterman & R. H. Freyberg. 1959. The genetics of gout and hyperuricemia—an analysis of nineteen families. J. Clin. Invest. **27:** 749.
11. Mugler, A. 1959. Un trouble humoral méconnu chez les goutteux: l'hyperoxalemia. Presse méd. **3:** 1.

EVALUATION OF PITUITARY-ADRENOCORTICAL FUNCTION IN PATIENTS WITH RHEUMATOID ARTHRITIS FOLLOWING STEROID THERAPY*

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One of the complications of corticosteroid therapy in patients suffering from rheumatoid arthritis is the poor tolerance of surgery occasionally shown by these patients while on, or after cessation of, a course of corticosteroids. In 1952, Fraser *et al.*[1] reported a case of death due to intractable vascular collapse following hip arthroplasty in a patient who had received cortisone up to two days before the operation. In one analogous case of Salassa *et al.*,[2] five months had elapsed between the termination of systemic steroid treatment and surgery. This observation suggested that the unresponsiveness of the pituitary-adrenocortical system to "stress" may persist for a long period after discontinuation of corticosteroids, which was strengthened by the pathological reports of Bennett.[3] Repeated injections of adrenocorticotropin have been proposed as a means of obviating this inadequacy in adrenocortical secretion. Ingle[4] had indeed demonstrated that the adrenocortical atrophy occurring during glucocorticoid administration is the result of the inhibition of adrenocorticotropin secretion by the adenohypophysis; this has been amply confirmed.[5,6] The adrenocortical responsiveness to adrenocorticotropin was actually found to be subnormal, sometimes for weeks, after corticosteroid therapy.[7-11] On the other hand, the concomitant use of adrenocorticotropin and corticosteroids is claimed to leave the adrenal cortex normally responsive to adrenocorticotropin once therapy is ended.[9,12] However, in one of the cases reported by Salassa *et al.*[2] this association had been used!

The restoration of a normal adrenocortical responsiveness to adrenocorticotropin obviously does not solve the problem. A proper assessment must be made of the extent to which corticosteroid therapy alters the ability of the adenohypophysis to react by an increased adrenocorticotropin release and secretion to stimuli such as "stress" situations or as decreased blood corticosteroid concentrations. Both types of stimuli, to be effective, require the integrity of hypothalamic structures besides a normally functioning adenohypophysis.[13,14]

Patients treated with corticosteroids have been studied by use of standardized "stress" situations, such as insulin-induced hypoglycemia and pyrogen-induced fever.[15] Amatruda *et al.*[16] observed a normal rise of the plasma corticosteroid concentration during insulin-induced hypoglycemia in patients who received adrenocorticotropin while on corticosteroids. Conversely, the intravenous injection of pyrogens failed to elicit the expected rise when patients had been

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only on corticosteroids.[17,18] The effect of surgery itself, known as inducing an increase in plasma corticosteroid concentration,[19] was examined by Marks *et al.*[20] who observed a normal response if the patients had been treated with adrenocorticotropin together with corticosteroids.

These are heroic procedures indeed, and only very few rheumatic patients were so studied. Another approach recently has become available, consisting of altering the normal adrenocorticotropin-cortisol feed back mechanism. A substance chemically related to amphenone, the 2-methyl-1,2-bis-(3-pyridyl)-1-propanone, commonly called SU 4885,[21] inhibits selectively at low concentrations the enzyme systems responsible for the oxidation of corticosteroids in position 11. This was established *in vivo* by Jenkins *et al.*[22] and Liddle *et al.*,[23] and *in vitro* by Tomkins.[24] Thus, as a result of administration of SU 4885 to normal subjects, 11-deoxycortisol is secreted by the adrenal cortex instead of cortisol; since 11-deoxycortisol is but a weak inhibitor of adrenocorticotropin secretion,[25] the latter increases as cortisol is cleared from the circulation. Consequently, steroidogenesis in the adrenal cortex is stimulated and more 11-deoxycortisol is produced; this steroid and its major metabolite, tetrahydro-11-deoxycortisol, are measurable by the colorimetric reaction of Porter and Silber.[26] Thus, after administration of SU 4885 there is a rise in the urinary excretion of the Porter-Silber reacting material. In FIGURE 1 it is apparent that such a rise is due essentially to the appearance in large amounts of tetrahydro-11-deoxycortisol in the urine.

This test thus makes possible an examination of the ability of the hypothalamopituitary-adrenocortical system to react to a decreased concentration of circulating cortisol by a rise in steroidogenesis. The test was carried out on 22 patients, of whom 20 had rheumatoid arthritis, 1 had gouty arthritis, and 1 had osteoarthritis. Sixteen of these 22 patients had been treated with various corticosteroids in varying amounts for periods extending from 3 weeks to 7 years. The corticosteroid treatment was interrupted 10 days to 17 months before the following tests were performed: (1) 2 intravenous infusions of 25 U.S.P. units of adrenocorticotropin in 500 ml. of isotonic saline, given over a period of 8 hours on 2 consecutive days; (2) 750 mg. of SU 4885 given by mouth every 4 hours for 48 hours. A minimum of 2 days separated both tests. Twenty-four-hour urine collections were obtained before and during these tests for measurement of 17-ketosteroid[27] and 17-hydroxycorticoid[28] excretion. Blood was drawn at the beginning and at the end of the SU 4885 test for the determination of plasma adrenocorticotropin concentration.[29]

A detailed account of these observations is published;[30] therefore only mean values for each group of patients will be discussed. As seen in TABLE 1, the base-line urinary excretion of 17-hydroxycorticoids in rheumatic patients, lower than in the control group, was the same whether the patients had been on corticosteroids or not. The administration of adrenocorticotropin accentuated the difference between the control group and the patients studied. Previous corticosteroid therapy did not appear to influence the mean response to adrenocorticotropin, but the excretion of 17-hydroxycorticoids by the untreated patients rose to 17.5 mg. per 24 hours on the second day of the SU 4885 test, as opposed to only 11.5 mg. per 24 hours by the corticosteroid-treated group. The lack of significant rise on the first day of the test, not readily interpreted, has been observed by other investigators.[31,32]

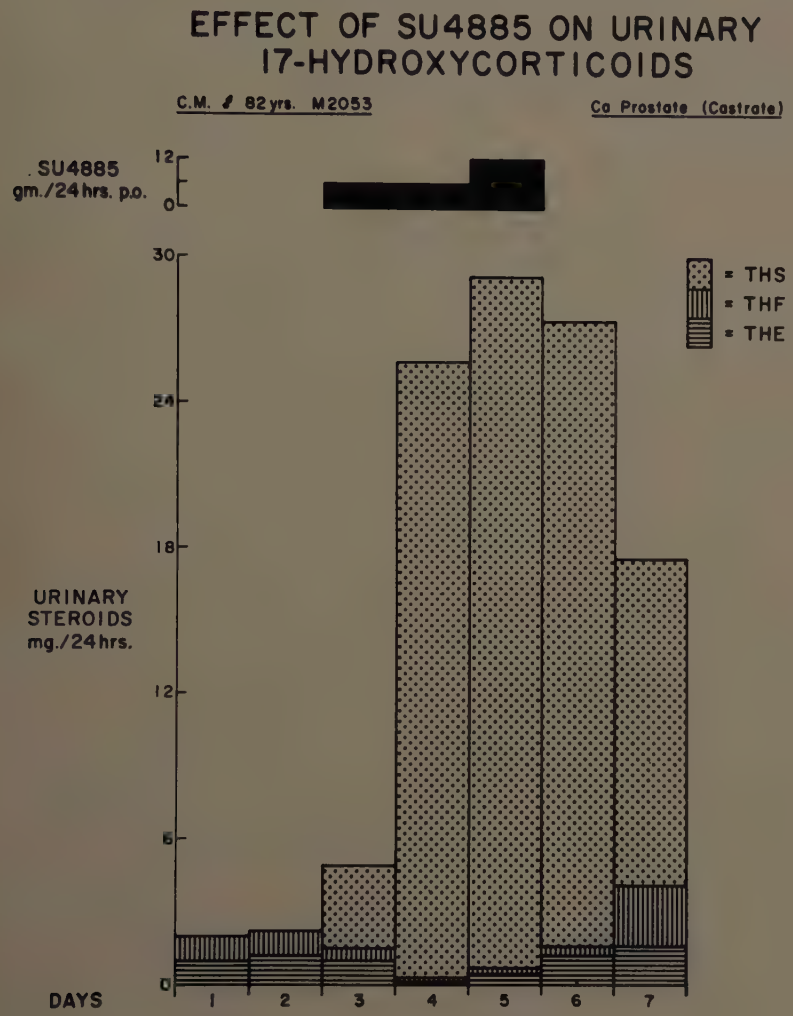

FIGURE 1. Reproduced from J. S. Jenkins *et al.* 1959. Brit. Med. J. **1:** 398.

TABLE 1

URINARY EXCRETION OF 17-HYDROXYCORTICOIDS (MG./24 HR.) IN RHEUMATIC PATIENTS BEFORE AND DURING ADMINISTRATION OF ADRENOCORTICOTROPIN OR SU 4885

	Base line	Adrenocorticotropin		SU 4885	
		Day 1	Day 2	Day 1	Day 2
Normal subjects (20)*	6	24.5	35	—	—
Patients untreated (6)*	4.1	14.3	14.4	5.9	17.5
Patients treated with corticosteroids (16)*	4.5	12.3	15.9	6.2	11.5

* Number of subjects in the group.

The 17-ketosteroid excretion was lowest in the group given corticosteroids, not only during the control days but also during the adrenocorticotropin and SU 4885 tests. The excretion values in the group of untreated patients were in turn lower than those of the control subjects, particularly in the female patients (TABLE 2).

The definitely low values of 17-hydroxycorticoids and 17-ketosteroids before and during adrenocorticotropin administration in patients with rheumatoid arthritis are not to be taken as specific for this kind of disease; similarly low levels are found in other chronically ill, middle-aged patients.[33]

The mean response of urinary steroids to the SU 4885 test in patients treated with corticosteroids was inferior to that obtained after maximal adrenocortical stimulation with exogenous adrenocorticotropin. This suggests a less adequate function of the hypothalamopituitary-adrenal axis in these patients. The measurement of plasma adrenocorticotropin concentrations allowed a sharper delineation of the defect. From undetectable concentrations before administration of SU 4885, plasma adrenocorticotropin rose to measurable levels after administration of the compound in 1 of the 6 untreated rheumatoid patients. It was elevated in 6 of the 16 patients who had been treated with corticosteroids. The highest concentration, 12 mU./100 cc., was measured in a patient who had been treated with 150 to 200 mg. of cortisone daily for 16 weeks until 2 weeks before the test was performed. Concentrations of 9, 8, and 7 mU./100 cc. were obtained from 3 patients who had received steroids for 3 to 4 years and whose steroid treatment had been terminated 3 to 4 weeks before the test. Concentrations of 4 and 5 mU./100 cc. were found in 2 patients who had taken prednisone for more than 2 years until 3 and 17 months prior to the SU 4885 test. Of interest is the fact that in 4 of these 6 patients the urinary 17-hydroxycorticoid excretion during adrenocorticotropin infusion was inferior to the mean values given in TABLE 1 for the group to which they belong.[30]

These elevated concentrations of adrenocorticotropin in the blood of corticosteroid-treated patients indicate that the hypothalamopituitary system recovers its ability to secrete adrenocorticotropin faster than the adrenal cortex regains its normal responsiveness to endogenous adrenocorticotropin. The observations of Gold *et al.* showed that a patient treated with adrenocorticotropin responded only normally to SU 4885,[34] in contrast with the poor response noted in 3 patients treated with steroids only.[35] This response is improved if the course of corticosteroid therapy is followed by injections of adrenocorticotropin.[32]

The persistence of poor adrenocortical response to adrenocorticotropin for a prolonged period of time is in contrast to the relatively faster recovery of the adrenocorticotropic secretion by the anterior pituitary gland. This strongly supports the advocated use of adrenocorticotropin at regular intervals whenever steroid therapy is instituted. However, a lasting failure of the hypothalamopituitary system should always be considered, as dramatically illustrated by Salassa's case.[2] It is for an appraisal of this possible failure that the SU 4885 test is of clinical value.

Summary

A study of the recovery of the pituitary-adrenocortical function after corticosteroid therapy has been carried out in 16 rheumatic patients. The patients

were examined by means of a standard adrenocorticotropin test as well as an SU 4885 test; in addition, blood adrenocorticotropin was measured at the beginning and at the end of the SU 4885 test. The results indicate that, following corticosteroid administration, the adrenocortical function is more impaired than the anterior pituitary function.

Acknowledgments

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TABLE 2

URINARY EXCRETION OF 17-KETOSTEROIDS (MG./24 HR.) IN RHEUMATIC PATIENTS BEFORE AND DURING ADMINISTRATION OF ADRENOCORTICOTROPIN OR SU 4885

Group	Sex	No. subjects	Mean age (yr.)	Base line	Adrenocorticotropin		SU 4885	
					Day 1	Day 2	Day 1	Day 2
Normal	M	10	30	14	25	32	—	—
	F	10	25	8	17	23	—	—
Untreated	M	3	57	12.0	21.8	23.6	12.7	30.8
	F	3	63	4.8	7.5	12.1	5.1	12.4
Treated	M	6	53	7.3	12.4	15.2	9.9	10.7
	F	10	60	4.2	7.3	8.5	3.3	9.0

References

1. FRASER, C. G., F. S. PREUSS & W. D. BIGFORD. 1952. Adrenal atrophy and irreversible shock associated with cortisone therapy. J. Am. Med. Assoc. **149:** 1542.
2. SALASSA, R. M., R. KEATING, JR. & R. G. SPRAGUE. 1953. Clinical aspects of suppression of adrenal cortical function after use of cortisone. Proc. Staff Meet. Mayo Clinic. **25:** 662.
3. BENNETT, W. A. 1954. Histopathologic alterations of adrenal and anterior pituitary in patients treated with cortisone. J. Bone Joint Surg. **36:** 867.
4. INGLE, D. J. 1938. The effects of administering large amounts of cortin on the adrenal cortices of normal and hypophysectomized rats. Am. J. Physiol. **124:** 369.
5. LEWIS, R. A., E. ROSENBERG & L. WILKINS. 1950. The effects of 17-hydroxy 11-dehydrocorticosterone upon the adrenals of normal and hypophysectomized rats maintained with adrenocorticotropin. Endocrinology. **47:** 414.
6. HOHLWEG, W. & U. LASCHET. 1959. Zur Frage des Cortison-und Corticotrophin-Wirkung auf das Hypophyse-Nebenniereurinden System. Acta Endocrinol. **32:** 437.
7. FORSHAM, P. H., G. W. THORN, T. F. FRAWLEY & L. W. WILSON. 1950. Studies on the functional state of the adrenal cortex during and following ACTH and cortisone therapy. J. Clin. Invest. **29:** 812.
8. CARREON, G. G., J. J. CANARY & L. H. KYLE. 1959. Adrenocortical function after long-term corticoid therapy. Clin. Research. **7:** 146.
9. BIERICH, J. R., I. KERSTEN & S. MARUEKTAD. 1959. Plasma corticosteroids and their

responsiveness to corticotropin after long term therapy with corticosteroids and corticotropin. Acta Endocrinol. **31:** 40.
10. Christy, N. P., E. Z. Wallace & J. W. Jailer. 1956. Comparative effects of prednisone and of cortisone in suppressing the response of the adrenal cortex to exogenous adrenocorticotropin. J. Clin. Endocrinol. Metabolism. **16:** 1059.
11. Krusius, F. E. & M. Oka. 1958. Effect of long-term treatment with corticosteroids on adrenocortical function. Studies in rheumatoid arthritis in the light of the response of plasma free and total 17, 21 dihydroxy 20 ketosteroids to intravenous corticotropin. Ann. Rheumatic Disease. **17:** 184.
12. Bondy, P. K., D. Hollingsworth, N. D. D'Esopo & G. V. Upton. 1958. Normal adrenal responsiveness in withdrawal hypoadrenalism. Clin. Research. **6:** 26.
13. Saffran, M. & J. Saffran. 1959. Adenohypophysis and adrenal cortex. Ann. Rev. Physiol. **21:** 403.
14. Sayers, G., E. S. Redgate & P. C. Royce. 1958. Hypothalamus, adenohypophysis and adrenal cortex. Ann. Rev. Physiol. **20:** 243.
15. Bliss, E. L., C. J. Migeon, K. Eiknes, A. A. Sandberg & L. T. Samuels. 1954. The effects of insulin, histamine, bacterial pyrogens and antabusalcohol reaction upon the levels of 17-hydroxycorticosteroids in the peripheral blood of man. Metabolism. **3:** 493.
16. Amatruda, T. T., Jr., D. Hollingsworth, N. D'Esopo, G. V. Upton & P. K. Bondy. 1958. Evidence of integrity of hypothalamus-pituitary-adrenal system after steroid withdrawal. Clin. Research. **6:** 253.
17. Farmer, T. A., Jr., J. A. Pittman & S. R. Hill, Jr. 1960. Studies on adrenocortical and anterior pituitary responsiveness. Clin. Research. **8:** 82.
18. Melby, J. C. 1959. Assessment of adrenocorticotropic activity with bacterial pyrogens in hypopituitary states. J. Clin. Invest. **38:** 1025.
19. Sandberg, A. A., K. Eiknes, L. T. Samuels & F. H. Tyler. 1954. The effects of surgery on the blood levels and metabolism of 17-hydroxycorticosteroids in man. J. Clin. Invest. **33:** 1509.
20. Marks, L. J., M. J. Donovan, F. J. Duncan & R. Karger. 1959. Adrenocortical response to surgical operations in patients treated with corticosteroids or corticotropin prior to surgery. J. Clin. Endocrinol. Metabolism. **19:** 1458.
21. Chart, J. J., H. Sheppard, M. J. Allen, W. L. Bencze & R. Gaunt. 1958. New amphenome analogues as adrenocortical inhibitors. Experientia. **14:** 15.
22. Jenkins, J. S., J. W. Meakin, D. H. Nelson & G. W. Thorn. 1958. Inhibition of of adrenal steroid 11 oxygenation in the dog. Science. **128:** 478.
23. Liddle, G. W., D. Island, E. M. Lance & A. P. Harris. 1958. Alterations of adrenal steroid patterns in man resulting from treatment with a chemical inhibitor of 11 β hydroxylation. J. Clin. Endocrinol. Metabolism. **18:** 906.
24. Tomkins, G. M. *cit.* by G. W. Liddle, *et al.*[23]
25. Eberlein, W. R. & A. M. Bongiovanni. 1956. Plasma and urinary corticosteroids in the hypertensive form of congenital adrenal hyperplasia. J. Biol. Chem. **223:** 85.
26. Porter, C. C. & R. H. Silber. 1950. A quantitative color reaction for cortisone and related 17, 21 dihydroxy 20 ketosteroids. J. Biol. Chem. **185:** 201.
27. Drekter, I. J., A. Heisler, G. R. Scism, S. Sterns, S. Pearson & T. H. McGavack. 1952. Determination of urinary steroids. I. Preparation of pigment-free extracts and simplified procedure for estimation of total 17-ketosteroids. J. Clin. Endocrinol. **12:** 55.
28. Reddy, W. S. 1954. Modification of the Reddy-Jenkins-Thorn method for the estimation of 17-hydroxycorticosteroids in urine. Metabolism. **3:** 489.
29. Nelson, D. H. & D. M. Hume. 1955. Corticosteroid secretion in the adrenal venous blood of the hypophysectomized dog as an assay for ACTH. Endocrinology. **57:** 184.
30. Meakin, J. W., M. S. Tantongco, J. Crabbe, T. F. Bayles & D. H. Nelson. Pituitary-adrenal function following long-term steroid therapy. Am. J. Med. In print.
31. Liddle, G. W., H. L. Estep, J. W. Kendall, Jr. & W. C. Williams, Jr. 1959. Clinical applications of a new test of pituitary reserve. J. Clin. Endocrinol. Metabolism **19:** 875.
32. Holub, D. A., J. W. Jailer, J. I. Kitay & A. G. Frantz. 1959. Direct and indirect estimation of pituitary adrenocorticotropin reserves in man following adrenal steroid therapy. J. Clin. Endocrinol. Metabolism. **19:** 1540.
33. Ulloa, A., H. L. Holley, M. C. Gautney, W. R. Starnes & S. R. Hill, Jr. 1959. Studies on the adrenocortical response to ACTH in patients with rheumatoid arthritis. Clin. Research. **7:** 308.
34. Gold, E. M., V. C. DiRaimondo, A. Sarner, S. Hane & P. H. Forsham. 1959. The evaluation of endogenous ACTH capacity by use of an adrenocortical 11 β hydroxylase inhibitor. Proc. 41st Meet. Endocrinol. Soc. Abstr. No. 117.
35. Gold, E. M., V. C. DiRaimondo & P. H. Forsham. 1960. Quantitation of pituitary corticotropin research in man by use of an adrenocortical 11 β hydroxylase inhibitor. Metabolism. **9:** 3.

PRODUCTION AND METABOLISM OF ADRENOCORTICOSTEROIDS IN CONNECTIVE TISSUE DISEASE*

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For many years there has been considerable speculation and investigation concerning the possible role of altered production or metabolism of adrenocorticosteroids in patients with connective tissue disease. Early investigators who concerned themselves with this problem were hampered both by inadequate methodology for the evaluation of adrenal cortical function and adrenocorticosteroid metabolism and by inadequate understanding of this group of diseases. During the past decade great strides have been made in both of these respects. Nevertheless, at the present time there is no general agreement as to the extent or even the existence of abnormalities in adrenocorticosteroid production or metabolism in patients with these diseases, and the role that such abnormalities, if indeed they do exist, may play in the pathogenesis of connective tissue diseases remains a matter for speculation.

Present-day knowledge of adrenocorticosteroid biogenesis and metabolism, although by no means complete, is extensive enough to make it apparent that the evaluation of adrenal cortical function and adrenocorticosteroid metabolism presents a complex problem. No longer may one think in the simple terms of overproduction or underproduction of "the hormone of the adrenal cortex," as was customary a few years ago. It now is well known that there are many adrenocorticosteroids, that these are formed by an orderly series of reactions whose rates are influenced by specific enzymes, that they vary considerably and even may be antagonistic in their metabolic effects, that each is metabolized in a characteristic way, that there may be simultaneous overproduction of one and underproduction of another, and that one may be metabolized at a normal rate while simultaneously another is metabolized at an abnormal rate. All of these factors must be taken into consideration in the evaluation of the status of adrenocortical function and steroid metabolism in a given disease state.

Awareness of the complexity of adrenal steroidogenesis and steroid metabolism has led to the realization that there is no real reason, a priori, to expect that steroid formation and metabolism must be the same in the various connective tissue diseases. Diseases of this group, although similar, are by no means identical; if abnormalities in steroid metabolism are of significance in the pathogenesis of these diseases, it perhaps would be more logical to expect some variation in the abnormalities observed in the different disease states. Only two of the connective tissue diseases, rheumatoid arthritis and rheumatic fever, have been studied extensively with regard to steroid production and metabolism. This communication will be concerned with a brief consideration

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of the findings in these diseases, with principal emphasis on those in rheumatic fever.

The concept of the general adaptation syndrome and the diseases of adaptation propounded by Selye[1] led to many investigations attempting to evaluate the status of pituitary-adrenocortical function in patients with connective tissue disease. Following the announcement by Hench *et al.*[2] of the dramatic effects of cortisone and adrenocorticotropic hormone (ACTH) in patients with rheumatoid arthritis, the subsequent confirmation of these by numerous investigators, and the demonstration of the corresponding effectiveness of these drugs in controlling the systemic symptomatology in patients with rheumatic fever, efforts to elucidate the role of the pituitary-adrenal axis in these diseases were intensified. Earlier studies[3-13] failed to detect evidence of any consistent abnormality in pituitary-adrenal function and gave rise to conflicting data and interpretations. However, recent investigations employing more specific techniques for evaluation of steroid production and metabolism have given clear indication that certain abnormalities in this regard do exist in patients with rheumatoid arthritis[14,15] and in patients with rheumatic fever.[16] Since the time, a few years ago, when one of us (V.C.K.) summarized the existing data indicating that consistent abnormalities in pituitary-adrenocortical function occur in patients with rheumatic fever,[16] additional studies in our laboratories and numerous other studies have provided considerable additional data relevant to the question of the status of adrenocortical function and steroid metabolism in rheumatic fever patients. However, despite the increased knowledge concerning this, there remain many unanswered and still unanswerable questions regarding the significance of the observations made. In this communication some of these pertinent questions are considered, and an attempt will be made to evaluate current information concerning each.

Is There Evidence of Decreased Adrenocortical Function?

Studies of urinary steroid excretion. One approach to the evaluation of adrenocortical function is the measurement of urinary excretion of adrenocortical steroids or their metabolites. Until recently, the methods used for such evaluation included only biological assay techniques and chemical determinations of urinary steroids, which were of limited value because they lacked either specificity or sensitivity.

Many of the earlier studies and some more recent ones have been concerned with the excretion of 17-ketosteroids in urine. Results have indicated clearly that in rheumatic disease there is not an increased excretion of these steroids, but their excretion has been reported to be normal,[3-5,11,14,15,17] low normal,[6,13,18,19] or subnormal.[7,12,20]

Several investigators[21-24] during the past few years have introduced techniques for the determination of urinary 17-hydroxycorticosteroids (17-OHCS) employing the Porter-Silber reaction.[25] Since the 17-OHCS group of adrenal steroids includes compound F (cortisol), which has been demonstrated to be the principal adrenocortical hormone in normal man,[26,27] determinations of urinary 17-OHCS might be expected to reflect more accurately the function of the adrenal cortex than do the older, less specific determinations of urinary corticoids.

In our laboratories[28] the 24-hour urinary 17-OHCS excretions in a group of children with active rheumatic fever were measured with the Glenn and Nelson technique[22] and compared with the values observed in normal children and adults. Normal children were found to excrete significantly smaller amounts of 17-OHCS than did normal adults, but there was no significant difference between normal children and children with active untreated rheumatic fever as regards their excretion of steroids of this type. Although it seems logical to interpret these data as indicating that adrenal function is normal in patients with active rheumatic fever, another interpretation might be possible. It is accepted generally that the adrenal cortex responds to "stress" stimuli by increasing its secretory activity, although the mechanisms involved are not understood completely. Severe "stress" such as that in acute illness or terminal conditions and even "psychic stress" cause elevations of plasma and urinary 17-OHCS concentrations.[26,29-32] During the first few days of illness with rheumatic fever, as in other acute illnesses, the plasma 17-OHCS concentrations are elevated.[33] One therefore might have expected to find increased urinary 17-OHCS excretion in these patients; however, they had entirely normal excretion values. If increased adrenocortical activity is present in these patients, it is not reflected by the excretion of 17-OHCS.

There have been conflicting reports regarding urinary corticoid excretion by rheumatoid patients. There have been no reports of increased excretion, but several investigators have reported urinary corticoid excretion to be normal[6,9,15,17] and several have reported it to be subnormal.[8,12,14,19,34,35] In a recent carefully controlled study[14] the urinary excretion of steroids by ambulatory rheumatoid arthritis patients was compared with that by hospitalized rheumatoid arthritics and by each of four control groups: ambulatory normal students, ambulatory older normal subjects, hospitalized normal subjects, and hospitalized chronic disease patients. The most interesting and perhaps significant observation made in this study was that the normal diurnal variation in corticoid excretion observed in all of the control groups did not occur in either the ambulatory or hospitalized rheumatoid arthritics. The greatest differences between the groups of rheumatoid arthritics and their corresponding control groups were evident in the 7 A.M. to 1 P.M. urine samples, reflecting the fact that in normal subjects this is the time the highest excretion values occur. During this period the corticoid excretions by the ambulatory and hospitalized rheumatoid patients were significantly lower, respectively, than those by the corresponding ambulatory and hospitalized control subjects. The mean 24-hour urinary excretion of 17-hydroxycorticosteroids also was significantly lower in ambulatory rheumatoid arthritics than in the ambulatory normal subjects; however, although the two groups with the lowest mean values for 24-hour urinary corticoid excretion were the groups of rheumatoid arthritics, the differences between the values in these groups and those in the other control groups were not statistically significant. No corresponding abnormalities of 17-ketosteroid excretion in either total 24-hour excretion or diurnal variation were observed in the rheumatoid arthritic groups. Consequently, the mean 17-ketosteroid/17-hydroxycorticosteroid concentration ratio was much greater in the urine of the ambulatory rheumatoid arthritics than in that of any other group, and this ratio was somewhat greater for the hospitalized rheumatoid

arthritics than for the corresponding control groups. The results in this study emphasize the importance of carefully selecting appropriate controls for studies of this type, and make apparent some possible explanations for discrepancies in results and interpretations presented by previous investigators.

Studies of steroid concentrations in blood. Another approach to the evaluation of adrenocortical function is through the measurement of plasma 17-hydroxycorticosteroid concentrations. From numerous studies of plasma 17-OHCS levels in normal and abnormal human subjects it now is well known that in most acute illnesses the plasma 17-OHCS concentration rises sharply during the acute phase of the illness and returns to normal as the patient recovers. Patients with various phases of rheumatic fever activity were studied[33] with regard to their plasma 17-OHCS concentrations as measured by the technique of Nelson and Samuels.[36] Included in these studies were 15 patients with "early acute" rheumatic fever (that is, within the first week of overt rheumatic activity). In these patients the mean plasma 17-OHCS concentration was 23 μg./100 ml., approximately twice the concentration (12 μg./100 ml.) observed in normal children. Although this value is greater than normal, the concentrations observed in this group are considerably lower than those observed in patients acutely sick with pneumonia, meningitis, or other acute severe illnesses.[26,29-31]

Also included in these studies were 31 patients with "well-established active" rheumatic fever (that is, patients who had the onset of symptomatology at least 2 weeks prior to the time of study). In this group of patients the mean plasma 17-OHCS concentration (5.9 μg./100 ml.) was significantly lower than that in the control children. Similarly, in 27 patients with chorea without other overt rheumatic symptomatology and in 87 patients with inactive rheumatic fever, the plasma 17-OHCS concentrations were significantly lower than in normal children. These low concentrations of plasma 17-OHCS have been found to persist in patients with inactive rheumatic fever for several years after the last evidence of rheumatic activity. Thus, in patients with rheumatic fever, except during the "early acute" phase, the plasma 17-OHCS concentrations are uniformly low. Although not much importance may be attributed to a single low plasma 17-OHCS value, consistently low values in the same individual or in a group of individuals in the same clinical classification are significant. This does not mean necessarily that in the rheumatic patient the adrenal cortex is unable to produce 17-OHCS as rapidly or in so great a quantity as in the normal individual. An alternative explanation might be that such individuals destroy or remove adrenal steroids from the blood more rapidly than they are replaced by the adrenal cortex. Another possibility is that the equilibrium state with regard to release of pituitary ACTH might be such as to provide insufficient stimulation to the adrenal cortex to maintain the circulating concentration of 17-OHCS as high as in the "normal" individual. Regardless of the explanation, it is apparent that homeostatic mechanisms that are adequate to maintain a certain level of circulating 17-OHCS in the "normal" individual are not adequate to maintain a similar level in these rheumatic patients.

Conflicting results have been reported concerning the concentrations of corticoids in peripheral blood in patients with rheumatoid arthritis. These

concentrations have been reported to be normal,[19,37-39] low,[40] and even high.[41] It has been reported that the diurnal variation in plasma corticoid concentration is less than normal,[42] with higher than normal values at 3:30 A.M. and a smaller than normal rise in the 7:00 A.M. specimen.

Studies of blood ACTH concentrations. The data presented above concerning plasma steroid concentrations in rheumatic fever patients does not eliminate the possibility that the anterior pituitary rather than the adrenal cortex is to be implicated in the failure of these patients to maintain usual circulating concentrations of 17-OHCS. To investigate this possibility a study was undertaken[43] in which the concentration of ACTH in the blood of a group of children with this disease was estimated by the adrenal ascorbic acid depletion technique of Sydnor and Sayers.[44]

In the group of untreated patients with active rheumatic fever there appeared to be a relationship between duration of rheumatic activity and concentration of ACTH in the blood. None of 5 children in whom the duration of symptomatology was less than 7 days had a sufficient concentration of blood ACTH to be detected by the technique employed. On the other hand, 19 of 21 determinations on the blood of patients who had been ill more than 7 days revealed measurable ACTH levels. Among these patients, who had been ill from 11 days to 25 weeks, there was no evidence of a trend toward either increasing or decreasing blood ACTH levels with increasing duration of illness. Although some of the children in this group had considerably higher blood levels of ACTH than others, there were no clinical findings that distinguished them from the remainder of the group.

Elevated blood ACTH concentrations also were detected in patients with inactive rheumatic fever. Blood samples were obtained from 10 weeks to 2½ years after the last evidences of rheumatic activity or discontinuation of therapy. Elevated concentrations of ACTH were found in 15 of 17 samples. One sample from each of 2 patients showed no detectable ACTH; in each of these 2 patients duplicate samples for assay had been obtained within a week. Each patient whose blood ACTH level was zero on one determination had a definitely elevated concentration of ACTH on the other determination, although in neither case was there any detectable change in the patient's clinical status.

These data indicate that in patients with rheumatic fever the blood ACTH concentration is related to the phase of rheumatic activity. Because of the limitations inherent in the method, the values reported may not represent precise determinations of the actual concentrations of circulating ACTH; nevertheless, the results are consistent and the pattern of change demonstrated throughout the course of illness with rheumatic fever is distinct. In children with "early acute" rheumatic fever (that is, during the first week after the onset of rheumatic symptomatology), as in normal children,[45] blood ACTH concentrations are not detectable by the method employed. However, in patients with "well-established" active rheumatic fever, inactive rheumatic fever, or chorea these concentrations are elevated. Conversely, as mentioned above, the plasma concentrations of 17-OHCS are higher than normal in patients with "early acute" rheumatic fever and lower than normal in patients with "well-established" active or inactive rheumatic fever.

The observation of an inverse relationship between blood ACTH and 17-OHCS concentrations in patients with untreated rheumatic fever is not compatible with the concept that the low levels of 17-OHCS in patients with this disease may be attributed to inability of the pituitary to elaborate ACTH. Rather, they are in conformity with the current concept of the humoral control of ACTH secretion. In accordance with this concept, the elevated ACTH levels in these patients may be considered secondary to the decreased corticosteroid levels, just as they are in adrenalectomized animals,[46,47] in patients with Addison's disease,[48-50] or in patients with congenital adrenal hyperplasia.[47]

The elevated blood ACTH levels in these rheumatic fever patients conceivably could be due to a primary abnormality in pituitary function. Since ACTH concentrations were not elevated during the initial phase of rheumatic fever, primary pituitary hypersecretion of ACTH appears unlikely; also, corticosteroid concentrations would be expected to be elevated, but were found to be depressed, when the ACTH concentrations were elevated. The formation (either initially by the pituitary, or subsequently by an altered metabolism) of an abnormal type of ACTH, with an impaired ability to stimulate steroidogenesis but with an unimpaired adrenal ascorbic-acid depleting activity, seems possible, but lacks substantiation.

The pattern of elevated 17-OHCS and undetectable ACTH concentrations during the "early acute" phase of rheumatic fever is consistent with that existing in patients with other acute illnesses. Such patients have elevated 17-OHCS concentrations;[26,29-31] yet no elevations of circulating ACTH have been observed in the few acutely ill patients studied.[43,48] It seems possible that an increased secretion of ACTH does occur temporarily, thereby effecting an increased concentration of circulating corticosteroids. These concentrations in turn may inhibit for a prolonged period any further ACTH secretion, so that a pattern of elevated steroid and undetectable ACTH concentrations results. This concept is compatible with the known relationship between the half life of ACTH and that of corticosteroids. The *in vivo* half life of endogenous ACTH is not greater than a few minutes;[51] on the other hand, the half life of corticosteroids normally is much longer[52-54] and, in acute rheumatic fever, is prolonged even further.[52]

Is There Evidence of Altered Steroidogenesis?

Most attempts at evaluation of pituitary-adrenal function in connective tissue disease employing direct measurement of steroid concentrations in blood and urine have concerned themselves only with measurements of 17-OHCS and 17-ketosteroids. As indicated in the preceding sections, it may be concluded that production and excretion of glucocorticoids certainly are not increased in these diseases, but are less than normal in rheumatic fever and possibly in rheumatoid arthritis. As emphasized by West,[15] the failure of severely ill rheumatoid arthritis patients to have even "high normal" values for urinary 17-OHCS excretion may indicate a relative deficiency in the production of glucocorticoids. There is, however, little to be gained by belaboring this point; even if it be conceded that patients with connective tissue disease have somewhat decreased production of glucocorticoids, it is obvious that this

in itself cannot be the crucial factor in the pathogenesis of their disease, since equally or more significantly decreased glucocorticoid production may occur not uncommonly in individuals with no evidence of connective tissue disease.

There has been much speculation on whether an abnormal "glucocorticoid/mineralocorticoid" ratio occurs and is of significance in the pathogenesis of connective tissue diseases. This question has not been resolved satisfactorily, primarily because adequate methodology has not been available. The complexity of the biochemical processes involved in adrenal steroidogenesis[55-57] now is well known. The number of different steroids resulting from adrenal steroidogenesis directly and from extra-adrenal metabolism of these compounds is staggering. Whereas it is convenient to consider steroids as being either "glucocortiocids" or "mineralocorticoids," in reality no such clear-cut distinction exists; many steroids exhibit considerable potency both as "glucocorticoids" and as "mineralocorticoids." Because of this, it is difficult to approach the problem of determining "glucocorticoid/mineralocorticoid" ratios or to evaluate the possible role that abnormalities in this ratio might play in the pathogenesis of connective tissue disease.

Those naturally occurring steroids measured as 17-OHCS may be considered to have primarily "glucocorticoid" activity. These can be measured with relative ease. On the other hand, aldosterone, the most potent known naturally occurring "mineralocorticoid," is present in blood in such low concentration that its measurement is not practicable. Aldosterone excretion in urine has been measured in patients with rheumatoid arthritis[58,59] and found to be within the normal range. Whether or not such data should be interpreted as indicating that aldosterone is produced in normal quantity by the rheumatoid arthritis patient is problematical.

Corticosterone has considerable "mineralocorticoid" activity, and is the second most abundant steroid of the adrenal cortical secretion in normal man, the ratio of cortisol to corticosterone concentration in normal young adults being approximately 3.5:1.[60] This steroid differs from cortisol in structure only by the absence of the hydroxyl group, which is present at the 17-α position in the latter. According to present concepts of adrenal steroidogenesis, the initial steps in the pathways for formation of cortisol and corticosterone are identical; only in the steps subsequent to formation of progesterone do these biogenetic pathways differ. In the normal individual, progesterone is converted mainly to cortisol by the introduction of hydroxyl groups successively at position 17 to form 17-α-hydroxyprogesterone, at position 21 to form 11-desoxycortisol (Reichstein's compound S), and position 11-β to form cortisol. However, some of the progesterone is not hydroxylated at the 17 position, but in the initial step is converted to 11-desoxycorticosterone by hydroxylation at position 21 and then to corticosterone by hydroxylation at the 11-β position.

As summarized recently by Axelrod and Goldzieher,[61] there have been clear demonstrations in specific abnormal states of spontaneously occurring decrease or absence of (1) 21 hydroxylating activity in the presence of adequate 11-β and 17-α hydroxylation,[62,63] (2) 11-β-hydroxylating activity in the presence of adequate 17-α and 21 hydroxylation,[64] and (3) combined 21 and 11-β-hydroxylating activity in the presence of adequate 17 hydroxylation.[65-67] However,

there have been no clear demonstrations of reduced 17-hydroxylating activity, so the syndromes that might result from this have not been defined. If 17-hydroxylating activity were absent but 11-β- and 21-hydroxylating activity remained intact, the major adrenal steroid secreted would be corticosterone. If both 17-α- and 11-β-hydroxylating activity were reduced but 21 hydroxylating activity remained intact, the major adrenal steroid secreted would be 11-desoxycorticosterone. Presumably in the latter case and probably in the former, aldosterone would be secreted in increased amount and, whether or not this occurred, there would result a decreased "glucocorticoid/mineralocorticoid" ratio.

In an attempt to determine whether or not corticosterone production is increased in patients with connective tissue disease, we have studied (E. R. Hughes, V. C. Kelley, and R. S. Ely, unpublished observations) plasma corticosterone concentrations in normal children and in children with rheumatic fever and rheumatoid arthritis by a modification[60] of the technique introduced by Sweat.[68,70] In normal children plasma corticosterone concentrations were

TABLE 1
PLASMA CORTICOSTERONE IN CHILDREN WITH RHEUMATIC DISEASE

	No. patients	No. tests	Corticosterone (μg./100 ml.)	P (vs. controls)
Control children (5 to 17 yr.)	31	31	3.2 ± 0.30	
Rheumatic fever				
Active	12	15	4.9 ± 0.32	0.01
Inactive	10	13	5.1 ± 0.71	<0.02
Rheumatoid arthritis	12	32	4.6 ± 0.48	<0.02

found to be substantially lower than 17-OHCS concentrations (3.2 versus 12.0 μg./100 ml.). As mentioned in a preceding section, the circulating 17-OHCS concentrations were low in rheumatic children. In contrast, as shown in TABLE 1, the corticosterone concentrations were elevated considerably in these subjects. In the control group the mean value was 3.2 μg./100 ml. In those children with active rheumatic fever it was 4.9, and in those with inactive rheumatic fever it was 5.1 μg./100 ml. Each of these mean values is significantly higher than that for the control group, as is that (4.6 μg./100 ml.) found in the group of patients with juvenile rheumatoid arthritis. It is noteworthy that, although these blood values are higher than normal in contrast to those for 17-OHCS, which were lower than normal in the same categories of patients, the abnormality in concentration found for each type of steroid is the same in both active and inactive phases of rheumatic disease.

On the basis of the 17-OHCS and corticosterone data, an altered steroidogenesis apparently exists in rheumatic subjects with simultaneously decreased production of the "glucocorticoid" 17-OHCS and increased production of the more "mineralocorticoid" compound, corticosterone.

Is there evidence of an altered adrenocortical responsiveness? The low plasma 17-OHCS levels in the presence of elevated endogenous ACTH levels would indicate that the adrenal does not respond to ACTH stimulation as adequately

in the rheumatic patient as it does in the normal individual. This also is suggested by the fact that rheumatic subjects, in contrast to normal individuals, do not produce increased amounts of 17-OHCS in response to salicylate administration even if this is carried to the point of producing salicylate intoxication.[70] In further study of this, we determined the response of plasma steroid concentrations to ACTH stimulation using a standard ACTH response test.[71] This test employed a dose of 25 I.U. ACTH, providing a maximal stimulus for adrenal steroidogenesis. TABLE 2 shows the 17-OHCS responses observed in rheumatic fever subjects. As shown in the table, the responses observed in patients with active rheumatic fever or with chorea did not differ from those observed in normal children. Thus, apparently rheumatic patients can respond adequately to maximal ACTH stimulation. Unfortunately, no data are avail-

TABLE 2
PLASMA 17-OHCS RESPONSE TO ACTH IN RHEUMATIC PATIENTS

	No. patients	Mean increase in 17-OHCS (μg./100 ml.)
Control	65	17 ± 1.4
Active rheumatic fever	35	23 ± 2.3
Chorea	9	21 ± 5.0

TABLE 3
PLASMA CORTICOSTERONE RESPONSE TO ACTH IN RHEUMATIC PATIENTS

	No. patients	Mean post-ACTH concentration (μg./100 ml.)	Mean increase (μg./100 ml.)
Control	28	6.1	2.9 ± 0.61
Rheumatic fever	6	11.1	5.7 ± 1.81

able concerning responses to small test doses of ACTH, which would give an indication as to their responsiveness to minimal ACTH stimulation.

Similar studies concerning the increase in corticosterone concentration in response to ACTH stimulation were performed in a few patients. The data are shown in TABLE 3. In the control group of 28 children the mean plasma corticosterone concentration after ACTH was 6.1 μg./100 ml., an increase of 2.9 μg./100 ml. over the pre-ACTH value. The rheumatic patients had a mean post-ACTH concentration of 11.1 μg./100 ml., representing an increase of 5.7 μg./100 ml. These relatively few data suggest that in rheumatic patients the production of corticosterone in response to ACTH is at least as great as it is in normal subjects and possibly greater, despite the elevated pre-ACTH concentrations.

Is there evidence of altered steroid metabolism? The evidence concerning altered pathways of adrenocorticosteroid metabolism has been reviewed recently by West[15] and will not be discussed in detail here. Suffice it to say that few detailed quantitative studies of steroid metabolism have been reported, but evidence has been advanced that rheumatoid arthritics differ from normal

subjects with regard to (1) the corticosteroid metabolites in urinary extracts, (2) the urinary steroid metabolites following the administration of 11-desoxycortisol, and (3) the steroid metabolites recovered from knee joints following intra-articular injection of cortisol. These findings may be of great significance, but are difficult to interpret. Little information of this type is available concerning rheumatic fever patients. In one such study, Lubschez[72] failed to demonstrate any significant differences between normal children and children with rheumatic fever with regard to urinary steroid metabolites.

Some evidence has been presented,[52] and additional unpublished evidence (V. C. Kelley, A. K. Done, T. A. Good, and R. S. Ely) indicates that the rate of metabolism of steroids, as reflected by the half life of exogenous steroid, is altered in patients with connective tissue disease. As shown in TABLE 4, the cortisol half life is markedly prolonged in children with active rheumatic fever

TABLE 4
CORTISOL HALF LIFE IN CHILDREN WITH ACTIVE RHEUMATIC FEVER

Patient No.	Age	Sex	Duration of illness (days)	Cortisol half life (min.)*
1	8	M	5	135
2	10	F	10	129
3	7	M	11	120
4	7	F	90	114
5	13	F	8	109
6	12	F	9	151
7	12	F	10	147
8	13	M	25	141
9	13	F	30	98
10	15	M	31	94
11	15	F	40	141
12	13	F	8 months	199

* Normal values in this laboratory: 7 to 10 years, 63.9 ± 4.5 min.; 12 to 14 years. 90.1 ± 3.9 min.; young adults, 98.3 ± 2.9 min.

as compared with that in "control" children of corresponding age. This prolongation of half life appears to persist as long as does intense activity of the rheumatic process, since there is no direct correlation between duration of rheumatic activity and cortisol half life in this group. The 2 children in the group who had normal half-life values (patients 9 and 10) were not acutely ill at the time of sampling, but had only mild rheumatic activity. Patients who were convalescent from acute attacks of rehumatic fever but had no residual evidences of rheumatic activity all had normal half-life values.

TABLE 5 shows the pretherapy cortisol half-life values observed in children with juvenile rheumatoid arthritis. The cortisol half life was prolonged in all of these patients. Only one of these children (patient 6) has had repeated studies. The initial study of this patient was done during a course of prolonged cortisone therapy and revealed a half life of 106 min. Eight months later, 3 months after steroid therapy was discontinued and at a time when the steroid-induced remission of clinical symptomatology persisted, the cortisol half life

was 102 min. However, after 8 months more had elapsed without therapy the patient returned with severe symptomatology of active rheumatoid arthritis and at this time her cortisol half life was 174 min., nearly twice normal.

We also have studied the half life of corticosterone in patients with connective tissue disease (E. R. Hughes, V. C. Kelley, and R. S. Ely, unpublished observations). Some of the data obtained are shown in TABLE 6. As indicated, a prolonged corticosterone half-life value was observed consistently in patients with rheumatic fever and other connective tissue diseases.

TABLE 5
CORTISOL HALF LIFE IN PATIENTS WITH JUVENILE RHEUMATOID ARTHRITIS

Patient No.	Age	Sex	Clinical status	Cortisol half life (min.)*
1	3	F	Moderate arthritis for 9 months	77
2	6	F	Severe arthritis for 2 years	180
3	6	F	Mild, early arthritis	83
4	8	F	Acutely ill for 2 weeks	111
5	10	M	Severe acute initial episode, 2 weeks' duration	133
6	14	F	Severe exacerbation, 6 weeks' duration	174

* Normal values for age (this laboratory): 1 to 6 years, 51.9 ± 2.4 min.; 7 to 10 years, 63.9 ± 4.5 min.; 12 to 14 years, 90.1 ± 3.9. min.

TABLE 6
CORTICOSTERONE HALF LIFE IN PATIENTS WITH CONNECTIVE TISSUE DISEASE

Group	No. subjects	Corticosterone half life (min.)
Control children	16	40 ± 1.8*
Active rheumatic fever	8	64 ± 7.0
Other connective tissue disease (active)	17	58 ± 4.0

* No variation with age has been observed.

Thus there appears to be a decreased rate of "metabolism" of both cortisol and corticosterone in patients with active connective tissue disease ("metabolism" is used here to include the summation of all processes involved in the removal of free steroid from the circulation, such as conjugation, excretion, degradation, utilization, tissue storage). These observations are important in interpreting some of the data presented earlier concerning blood and urinary steroid concentrations. For example, the low 17-OHCS plasma concentrations observed in patients with rheumatic fever obviously cannot be attributed to a more rapid than normal removal of these steroids from the circulation. Similarly, the simultaneous occurrence of decreased 17-OHCS and increased corticosterone plasma concentrations in these patients probably cannot be attributed to differences in the rates of removal of these steroids from circulation, since both are removed more slowly than normal.

Summary

Several pertinent questions are posed regarding production and metabolism of adrenocorticosteroids in connective tissue disease: (1) Is there evidence of decreased adrenocortical function? (2) Is there evidence of altered steroidogenesis? (3) Is there evidence of an altered adrenocortical responsiveness? (4) Is there evidence of altered steroid metabolism? An attempt has been made to evaluate current information with regard to each of these questions.

It appears that neither adrenocortical function nor steroid metabolism is completely normal in patients with connective tissue disease, but the abnormalities that exist may be quite subtle and difficult to demonstrate unequivocally. There is as yet no clear demonstration that these abnormalities are of significance in the pathogenesis of connective tissue disease; however, this remains a distinct possibility that deserves continuing investigation.

References

1. Selye, H. 1946. The general adaptation syndrome and the diseases of adaptation. J. Clin. Endocrinol. **6:** 117.
2. Hench, P. S., E. C. Kendall, C. H. Slocumb & H. F. Polley. 1949. Effect of hormone of adrenal cortex (17-hydroxy-11-dehydrocorticosterone: compound E) and of pituitary adrenocorticotropic hormone on rheumatoid arthritis. Preliminary Rept. Proc. Staff. Meet. Mayo Clin. **24:** 181.
3. Thorn, G. W., T. B. Bayles, B. F. Massell, P. H. Forsham, S. R. Hill, Jr., E. Smith, III, & J. S. Warren. 1949. Studies on the relation of pituitary-adrenal function to rheumatic disease. New Engl. J. Med. **241:** 529.
4. Davison, R. A., P. Koets & W. C. Kuzell. 1947. Excretion of 17-ketosteroids in ankylosing spondylarthritis and in rheumatoid arthritis, preliminary report. J. Clin. Endocrinol. **7:** 201.
5. Desmarais, M. H. L. 1949. The neutral 17-ketosteroids in rheumatoid arthritis and spondylitis. Ann. Rheumatic Diseases. **8:** 296.
6. Hench, P. S., E. C. Kendall, C. H. Slocumb & H. F. Polley. 1950. Effects of cortisone acetate and pituitary ACTH on rheumatoid arthritis, rheumatic fever and certain other conditions: a study in clinical physiology .A.M.A. Arch. Internal Med. **85:** 545.
7. Freund, H. A., D. H. Basinski & R. B. Scott. 1950. 17-Ketosteroid excretion in rheumatoid arthritis. J. Michigan State Med. Soc. **49:** 1076.
8. Howard, R. P., E. H. Venning & G. H. Fisk. 1950. Studies of adrenocortical and hypophyseal function and the effects thereon of testosterone and pregnenolone therapy. Can. Med. Assoc. J. **63:** 340.
9. Staub, P. L., J. W. Menthe, S. S. Nelson & H. Cohn. 1950. Excretion of 11-oxycorticosteroids in paraplegic and rheumatoid arthritis patients. J. Clin. Invest. **29:** 349.
10. Thorn, G. W. & T. B. Bayles. 1949. Current therapeutics: XXII. Pituitary adrenal function and rheumatic disease. Practitioner. **163:** 365.
11. Wolfson, W. Q., H. S. Guterman, R. Levine, C. Cohn, H. D. Hunt & E. F. Rosenberg. 1949. An endocrine finding apparently characteristic of gout: very low urinary 17-ketosteroid excretion with clinically normal androgenic function. J. Clin. Endocrinol. **9:** 497.
12. Venning, E. H., L. G. Johnson & B. Rose. 1951. Adrenal cortical function in rheumatoid arthritis and asthma. Proc. Second Clin. ACTH Conf. **2:** 49.
13. Marti, M. 1951. Die Chromatographische Trennung und Bestimmung der 17-Ketosteroide in Urin. Methodik und Klinische-Experimentelle Anwendung. Helv. Med. Acta. **18:** 215.
14. Hill, S. R., Jr., H. L. Holley, A. Ulloa, W. R. Starnes, L. L. Hibbitt & J. H. McNeil. 1959. Studies on adrenal cortical activity in patients with rheumatoid arthritis: The diurnal pattern and twenty-four hour levels of urinary total 17-hydroxycorticosteroids and 17-ketosteroids. Arthritis and Rheumatism. **2:** 114.
15. West, H. F. 1957. Corticosteroid metabolism and rheumatoid arthritis. Ann. Rheumatic Diseases. **16:** 173.
16. Kelley, V. C. 1955. Rationale for hormone therapy in rheumatic fever. Ann. N. Y. Acad. Sci. **61**(2): 369.
17. Fischer, F. 1955. Corticotropin and cortisone in rheumatoid arthritis. Acta Med. Scand. (Suppl. 305) **152:** 7.

18. Birke, G. & L. O. Plantin. 1954. Studies on urinary 17-ketosteroids. Acta Med. Scand. (Suppl. 291) **148:** 8.
19. Birke, G. & L. O. Plantin. 1957. Adrenal cortical activity and steroid metabolism in rheumatoid arthritis. Acta Rheumatic Scand. **3:** 59.
20. Kellie, A. E. & A. P. Wade. 1953. Urinary steroid excretion in rheumatoid arthritis: changes in ketonic and non-ketonic fractions during hormone therapy. Brit. Med. J. **2:** 594.
21. Reddy, W. J., D. Jenkins & G. W. Thorn. 1952. Estimation of 17-hydroxycorticosteroids in urine. Metabolism. **1:** 511.
22. Glenn, E. M. & D. H. Nelson. 1953. Chemical method for the determination of 17-hydroxycorticosteroids and 17-ketosteroids in urine following hydrolysis with β-glucuronidase. J. Clin. Endocrinol. and Metabolism. **13:** 911.
23. Vestergaard, P. 1953. Investigations on the estimation of 17-hydroxycorticosteroids in urine using the Porter-Silber reaction. Acta Endocrinol. **13:** 911.
24. Smith, R. W., R. C. Mellinger & A. A. Patti. 1954. Modifications of the Reddy procedure for 17-hydroxycorticoids in urine. J. Clin. Endocrinol. and Metabolism. **14:** 336.
25. Porter, C. C. & R. H. Silber. 1950. A quantitative color reaction for cortisone and related 17, 21-dihydroxy-20-ketosteroids. J. Biol. Chem. **185:** 201.
26. Nelson, D. H., L. T. Samuels, D. G. Willardson & F. H. Tyler. 1951. The levels of 17-hydroxycorticosteroids in peripheral blood of human subjects. J. Clin. Endocrinol. **11:** 1021.
27. Mason, H. L. 1950. Urinary excretion of steroids during administration of ACTH. *In* Proceedings of the First Clinical ACTH Conference, J. R. Mote, Ed. : 168. Blakiston. Philadelphia, Pa.
28. Ely, R. S., L. E. Ainger, J. R. Seely, A. K. Done & V. C. Kelley. 1955. Studies of 17-hydroxycorticosteroids. X. Urinary excretion of 17-hydroxycorticosteroids in patients with rheumatic fever. J. Clin. Endocrinol. and Metabolism. **15:** 523.
29. Klein, R., M. D. Papadatos & J. Fortunato. 1953. Serum corticoids in children under stress. J. Pediat. **43:** 385.
30. Kelley, V. C. 1955. Studies of pituitary-adrenal hormones in children in health and disease. Pediatrics. **15:** 1.
31. Done, A. K., R. S. Ely & V. C. Kelley. 1958. Studies of 17-hydroxycorticosteroids. XIV. Plasma 17-hydroxycorticosteroid concentrations at death in human subjects. A.M.A. Am. J. Diseases Children. **96:** 655.
32. Hetzel, B. S., W. W. Schottstaedt, W. J. Grace & H. G. Wolff. 1954. Changes in urinary 17-hydroxycorticosteroid excretion during stressful life experiences in man. (Abstr.) J. Clin. Endocrinol. and Metabolism. **14:** 805.
33. Kelley, V. C., R. S. Ely, A. K. Done & L. E. Ainger. 1955. Studies of 17-hydroxycorticosteroids. VI. Circulating concentrations in patients with rheumatic fever. Am. J. Med. **18:** 20.
34. Norymberski, J. K., R. D. Stubbs & H. F. West. 1953. Assessment of adrenocortical activity by assay of 17-ketogenic steroids in urine. Lancet. **1:** 1276.
35. Appleby, J. I. & J. K. Norymberski. 1955. Urinary excretion of 17-hydroxy-20-oxosteroids in normal health and in rheumatoid arthritis. Ann. Rheumatic Diseases. **14:** 172.
36. Nelson, D. H. & L. T. Samuels. 1952. A method for the determination of 17-hydroxycorticosteroids in blood: 17-hydroxycorticosterone in the peripheral blood. J. Clin. Endocrinol. and Metabolism. **12:** 519.
37. Nelson, D. H., L. T. Samuels, D. G. Willardson & F. H. Tyler. 1951. The levels of 17-hydroxycorticosteroids in *peripheral* blood of human subjects. J. Clin. Endocrinol. **11:** 1021.
38. Birke, G. 1953. Aspects of the steroid metabolism in rheumatoid arthritis and rheumatic fever. Summary of the communications. VIII. Congrès International des Maladies Rhumatismales. Méd. et Hyg. (Genève). : 50.
39. Perkoff, G. T., A. A. Sandberg, D. H. Nelson & F. H. Tyler. 1954. Clinical usefulness of determination of circulating 17-hydroxycorticosteroid levels. A.M.A. Arch. Internal Med. **93:** 1.
40. Ceresa, F. & C. Reyneri. 1953. Adrenocorticotrophic activity of plasma in subjects suffering from rheumatoid arthritis. Acta Endocrinol. **12:** 85.
41. Freeman, S., J. X. Wheeler & H. W. Hoegemeier. 1956. Free reducing and hydrocortisone-like steroids in human plasma. A.M.A. Arch. Internal Med. **97:** 45.
42. Warren, J. E. 1956. Diurnal plasma corticoid studies and their relation to morning stiffness in rheumatoid arthritis. (Abstr.) Ann. Rheumatic Diseases. **15:** 392.
43. Brill, A. B., R. S. Ely, A. K. Done, L. E. Ainger & V. C. Kelley. 1956. Blood adrenocorticotropin (ACTH) in children with rheumatic fever. J. Clin. Endocrinol. and Metabolism. **16:** 262.

44. Sydnor, K. L. & G. Sayers. 1952. A technic for determination of adrenocorticotrophin in blood. Proc. Soc. Exptl. Biol. Med. **79:** 432.
45. Sydnor, K. L., V. C. Kelley, R. B. Raile, R. S. Ely & G. Sayers. 1953. Blood adrenocorticotrophin in children with congenital adrenal hyperplasia. Proc. Soc. Exptl. Biol. Med. **82:** 695.
46. Gemzell, C. A., D. C. Van Dyke, C. A. Tobias & H. M. Evans. 1951. Increase in the formation and secretion of ACTH following adrenalectomy. Endocrinology. **49:** 325.
47. McCann, S. M. & K. L. Sydnor. 1954. Blood and pituitary adrenocorticotrophin in adrenalectomized rats with hypothalamic lesions. Proc. Soc. Exptl. Biol. Med. **87:** 369.
48. Sydnor, K. L., G. Sayers, H. Brown & F. H. Tyler. 1953. Preliminary studies on blood ACTH in man. J. Clin. Endocrinol. and Metabolism. **13:** 891.
49. Upson, M., Jr. 1951. Studies on Adrenocorticotropic Hormone in Human Serum. Thesis Graduate School. Univ. Minn. Minneapolis, Minn.
50. Taylor, A. B., A. Albert & R. G. Sprague. 1949. Adrenotrophic activity of human blood. Endocrinology. **45:** 335.
51. Sydnor, K. L. &. G. Sayers. 1953. Biological half-life of endogenous ACTH. Proc. Soc. Exptl. Biol. Med. **83:** 729.
52. Done, A. K., R. S. Ely, L. J. Olsen & V. C. Kelley. 1955. The *in vivo* half-life of exogenous hydrocortisone in patients with rheumatic fever. Metabolism. **4:** 416.
53. Brown, H., D. G. Willardson, L. T. Samuels & F. H. Tyler. 1954. 17-hydroxycorticosteroid metabolism in liver disease. J. Clin. Invest. **33:** 1524.
54. Peterson, R. E. & J. B. Wyngaarden. 1955. Metabolism of hydrocortisone and cortisone in man. Ann. N. Y. Acad. Sci. **61**(2): 297.
55. Pincus, G. 1954. The biosynthesis of adrenal steroids. Progr. in Allergy. **4:** 198.
56. Hechter, O. & G. Pincus. 1954. Genesis of the adrenocortical secretion. Physiol. Revs. **34:** 459.
57. Pincus, G. 1955. The biosynthesis of adrenal steroids. Ann. N. Y. Acad. Sci. **61**(2): 283.
58. Venning, E. H., J. C. Beck, I. Dyrenfurth & C. J. P. Giroud. 1955. Studies on the excretion of the sodium-retaining corticoid. J. Clin. Endocrinol. and Metabolism. **15:** 853.
59. Ward, L. E. & P. S. Hench. 1955. Effects of aldosterone (electrocortin), 9 alpha-fluorohydrocortisone acetate, and 1-dehydrocortisone (metacortandracin) in rheumatoid arthritis. Ann. N. Y. Acad. Sci. **61**(2): 620.
60. Ely, R. S., E. R. Hughes & V. C. Kelley. 1958. Studies of adrenal corticosteroids. I. Estimation of plasma corticosterone and cortisol. J. Clin. Endocrinol. and Metabolism. **18:** 190.
61. Axelrod, L. R. & J. W. Goldzieher. 1960. The metabolism of 17α-hydroxyprogesterone and its relation to congenital adrenal hyperplasia. J. Clin. Endocrinol. and Metabolism. **20:** 238.
62. Herrmann, W. & L. Silverman. 1957. Excretion of pregnane-3α,17α,20α-triol in pregnancy. J. Clin. Endocrinol. and Metabolism. **17:** 1482.
63. Eberlein, W. R. & A. M. Bongiovanni. 1955. Congenital adrenal hyperplasia with hypertension: unusual steroid pattern in blood and urine. J. Clin. Endocrinol. and Metabolism. **15:** 1531.
64. Masuda, M. 1957. Urinary ketosteroid excretion patterns in congenital adrenal hyperplasia. J. Clin. Endocrinol. and Metabolism. **17:** 1181.
65. Jailer, J. W., J. J. Gold, R. Vande Wiele & S. Lieberman. 1955. 17α-hydroxyprogesterone and 21-desoxyhydrocortisone; their metabolism and possible role in congenital adrenal virilism. J. Clin. Invest. **34:** 1639.
66. Bongiovanni, A. M., W. R. Eberlein & J. Cara. 1954. Studies on the metabolism of adrenal steroids in the adrenogenital syndrome. J. Clin. Endocrinol. and Metabolism. **14:** 409.
67. Bloch, E., R. I. Dorfman & G. Pincus. 1957. Conversion of acetate to C_{19} steroids by human adrenal gland slices. J. Biol. Chem. **224:** 737.
68. Sweat, M. L. 1954. Silica gel microcolumn for chromatographic resolution of cortical steroids. Anal. Chem. **26:** 1964.
69. Sweat, M. L. 1954. Sulfuric acid—induced fluorescence of corticosteroids. Anal. Chem. **26:** 773.
70. Done, A. K., R. S. Ely & V. C. Kelley. 1958. Salicylates and the pituitary-adrenal system. Metabolism. **7:** 52.
71. Ely, R. S., R. B. Raile, P. F. Bray & V. C. Kelley. 1954. Studies of 17-hydroxycorticosteroids. IV. Evaluation of a standard ACTH-17-hydroxycorticosteroid response test in children. Pediatrics. **13:** 403.
72. Lubschez, R. 1955. Identification of urinary 17-ketosteroids in rheumatic fever. Pediatrics. **15:** 537.

AN ATTEMPT TO RATIONALIZE THERAPY OF RHEUMATIC DISEASE

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The clinician responsible for treating the diseases of connective tissue listens to the scientific reports as presented in this symposium and wonders how this newer knowledge may help him in his difficult task. A prominent physicist recently asked: "How can you be sure of anything in medicine? You have no constants, and only an infinite number of variables with which to work." To those in the exact sciences, the approximations, estimates, and conjectures used in the biological sciences seem a poor substitute for the formulas based on constants that are available to them. All workers in the field of biology in general, and medicine in particular, are accustomed to "usually" or "rarely" instead of "always" or "never," and the number of variables reaches infinity in dealing with the total organism of man, as does the clinician. How, then, can a rationale for therapy be proposed for a heterogeneous group of connective tissue diseases of unknown etiology that seem at least slightly different in every victim? Obviously, such an assignment is impossible. This presentation *cannot* be scientific. It must be a patchwork of conjecture, remotely based on scattered facts, empiricism, trial-and-error, and experience. It can be justified only as a working hypothesis formulated by a clinician who feels the need for a framework upon which to assemble widely scattered bits of knowledge. The interstices can be filled only with conjecture, but the physician *must* have some over-all plan for a rationalization of the various treatment measures that together may help his patient combat the connective tissue disorder.

If these disorders are actually diseases of the immune mechanism of the body, with abnormal macroglobulins deposited in the connective tissues acting as autoantibodies, it can be understood why such conditions tend to be chronic and progressive, and the variations in severity throughout the course might even be explained, but still there is no clue to the etiological factor initiating the abnormal production and deposition of macroglobulins. It is possible that these abnormal proteins are merely accumulated waste products of the inflammation, comparable to amyloid or analogous to gouty tophi, which contaminate the tissues and make it still more difficult for the organism to cope with the disease.

It still is possible that infection, perhaps from a yet unidentified virus, initiates the abnormal immune reaction. This appears plausible on the basis of the sequence of events observed in rheumatic fever, but the similar connective tissue lesions produced by the deliberate sensitization of experimental animals and the development of connective tissue diseases in man in the apparent absence of clinical infection leave important gaps in this line of conjecture.

In the case histories of hundreds of individuals with rheumatoid arthritis, systemic lupus erythematosus, polyarteritis, and dermatomyositis it is noteworthy that in many the onset had occurred after some type of infection; in others the disease followed use of a drug; in still others it developed after a

harrowing emotional crisis, a prolonged grueling experience, severe exposure, or even malnutrition. Selye has grouped all such factors as nonspecific stresses and has popularized the idea that if any one or a group of stressing factors overcomes the adaptive resistance of the individual, a breakdown of his adaptive mechanism (which includes, of course, the immune mechanism) will occur.

The pathogenesis of this hypothetical breakdown of normal adaptation is still the subject of much speculation, and Selye's tenuous explanations have had to be revised repeatedly and altered to meet all challengers. It is not the purpose of this presentation to enter into the pros and cons of the adaptation syndrome as an explanation of the pathogenesis of connective tissue disease states, but it *is* expedient that the clinician accept the general idea, at least tentatively, since he does find stresses preceding the onset or exacerbations of connective tissue diseases in a majority of such patients.

The basic program of management of connective tissue diseases, developed after years of trial-and-error experience, consists of adequate rest to overcome fatigue, avoidance of chilling, prompt treatment of infections, proper diet, mental relaxation to overcome tensions, and adequate exercise or other physical measures to prevent decreased circulation and metabolism that would add to tissue breakdown or deformity. To paraphrase Selye, these methods of treatment might well be grouped under the common denominator of "antistresses," that is, measures to prevent further stress. These simple measures have stood the test of time and, in milder cases, may be sufficient treatment themselves.

The rationale for the so-called symptomatic measures is fairly obvious. Measures to combat anemia, to encourage rest and sleep, to allow mental relaxation, to encourage proper nutrition, and to relieve pain, whether or not these agents are drugs, help the individual to live on in better condition to cope with the disease. No one would deny the importance of such measures in the treatment program, although there is much disagreement concerning their actual effectiveness in the presence of active inflammatory disease. This is particularly true of hematinics, tranquilizers, and analgesics. All such symptomatic measures also may be grouped as agents helping to prevent further stress or to bolster the victim in a nonspecific manner.

The place of steroid therapy also might well be here. The suppression of many of the overt manifestations of connective tissue inflammation certainly has a beneficial effect on the morale of the victim, allowing better nutrition, relaxation, activity, and sleep. Fundamentally, however, it may be rationalized that the effects on the immune mechanism, including lysis of lymphocytes and perhaps of plasma cells, release of antibody, and decreased formation of additional antibody, are beneficial, as is the anti-inflammatory effect at the tissue level. Cortisone, hydrocortisone, and the newer synthetic cortisone derivatives represent the most potent nonspecific antirheumatic agents thus far known, but they apparently have no effect on the cause or causes of connective tissue disorders or on the *basic* disease process itself. It has been all too frequently obvious that the lesions of rheumatoid arthritis produce continued joint destruction under the cover of steroid therapy. Furthermore, it appears that steroid therapy has little effect on advancing lesions of polyarteritis, dermatomyositis, or lupus erythematosus, although it prevents acute clinical exacerbations.

Steroid therapy for the so-called collagen diseases might be analogous to digitalis for cardiac diseases. Digitalis, also a steroid, has no direct effect on the lesions of heart disease, nor does it halt their progress. It does not affect the cause of the lesions, but produces an amelioration of symptoms and signs by improvement in cardiac function in much the same way that the cortisones produce an improvement of symptoms and musculoskeletal function as long as administration is continued.

Many patients with rheumatoid arthritis or systemic lupus erythematosus are working daily, in spite of continued disease, who before taking cortisone hardly could care for their most fundamental personal needs. With better muscle power, decreased pain, and better nutrition they can live more normally and, certainly, more enjoyably despite their chronic incurable disease. This seems quite analogous to cardiac patients who could not move about without dyspnea, precordial pain, and marked edema until digitalis enabled them to resume and continue their regular jobs.

Both cortisone and digitalis may be considered "pharmaceutical crutches," either to help in an emergency or for protracted use when the patient cannot continue a reasonably active existence without them. In chronic conditions crutches should not be discarded, nor should a medicinal agent that helps to "prop up" the victim be withdrawn suddenly. Those who use crutches may expect some falls, those who need digitalis for a failing heart may go into failure in spite of the drug, and the cortisone-treated patient likewise can expect possible progression of his disease or undesirable side effects in spite of continued therapy. These agents may not add years to the life of the victim, but they do add life to his years.

Perhaps this analogy will also help to emphasize the fact that the use of steroid therapy is not warranted for all patients with connective tissue disorders. The mere presence of stiffness, pain, swelling, recurrent low-grade fever, and skin manifestations does not justify cortisone therapy per se. Only when the disease is severe enough to interfere seriously with the patient's normal functions, that is, "rheumatic decompensation," or when more conservative measures have proved inadequate, is the calculated risk of steroid therapy justified. Experience has shown that women over forty years of age are more likely to have difficulties from use of the steroids; therefore, their need of such aid must be greater than that of others. Once such therapy has been undertaken, it is extremely difficult to withdraw it.

If we may oversimplify the Selye concept by thinking of the connective tissue breakdown as similar to a blown fuse in an electric circuit produced by an overload on the line, regardless of cause, we may liken steroid therapy to the artificial substitution of a much stronger fuse. If a simple overload is responsible for a break in the circuit, the stronger fuse will serve nicely, but if a short circuit is responsible, the building may catch fire. A perforating or hemorrhaging peptic ulcer, angiitis, or spinal fractures from severe osteoporosis are certainly as dangerous to the body as fire to a building. Usually patients get along safely with the double-strength cortisone fuse, but it is impossible to be certain in any given case whether a simple overload or a serious short circuit caused the break.

Steroids may be likened also to automobiles, not only because newer, more

powerful and more streamlined models appear almost yearly—all of them useful for differing needs—but also because they are still potentially injurious, particularly when used recklessly. In spite of this potential danger, however, no thinking clinician would wish to abandon steroid use any more than he would wish to trade his car for a horse and buggy. Just as he must learn to drive carefully for his own safety and that of others, he must learn to use these valuable agents as safely and effectively as possible. Just as he must decrease his driving speed to avoid accidents, he must drive slowly with steroid therapy, using minimum doses and remaining ever alert for danger signals. Steroid therapy is here to stay, as is the automobile, and there is no longer a question of whether to employ steroids; the question is when and how to utilize them.

It does seem reasonable, when the marked connective tissue inflammation is confined chiefly to one or two joints, tendon sheaths, or bursae, that steroid should be injected locally for palliation before systemic steroid therapy is used. One puts a drop of oil where a mechanism squeaks, instead of flooding the whole machine with oil.

Construction of a rationale for the use of drugs such as salicylates (for effects other than analgesic), the chloroquines and similar antimalarials, phenylbutazone, gold salts, colloidal sulfur, bee venom, foreign protein, or the use of fever therapy, vaccines, para-amino benzoate, nitrogen mustard, and a multitude of other agents used empirically for rheumatoid arthritis and other connective tissue disorders for many years, calls for extreme ingenuity on the part of the clinician assigned to such a task. What possibly could there be in common between these heterogenous and extensively used agents? What, indeed, except that all are potenially toxic? Do they all poison a common enzyme system? Does their toxicity itself stimulate a jaded "adaptation" or immune mechanism?

It would appear from clinical results that the connective tissue diseases often can be ameliorated at least partially by what apparently are stressing agents themselves. It is well known that many of these disorders go into remission in the presence of active intercurrent fevers such as those in pneumonia or other acute infections, or in acute liver disease, pregnancy, or surgical operations. Yet, after such acute additional stresses are removed, the connective tissue disease usually reverts to its previous severity or becomes even worse.

Perhaps long-continued therapy with toxic drugs in doses just short of overt toxicity is a type of counterirritation or fighting-fire-with-fire treatment, a continual whipping-up-the-tired-horse in the hope that the causative agent will be eliminated in the time gained. Whether such agents actually work as continued and measured counterstresses is, of course, entirely unknown.

To those who feel as all rational scientists should feel, that these ideas are tenuous, it should be stated that the clinician who built this house of cards is not proud of it. When the scientists replace each card with a building stone of fact, none will be happier than the clinician, who must explain to his patient *why* he administers each item of the therapeutic program for his connective tissue disease.

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